CCXV. A SURVEY OF ANTHOCYANINS. V

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Employing the methods previously described [Robinson & Robinson, 1931; 1932; 1933; 1934], further flower petals and permanently pigmented leaves have been examined in order to determine the anthocyanin type. Comments on these results together with those previously recorded will be submitted later. The only new points of technique are the following.

A characteristic property of peonin is that solutions in aqueous sodium carbonate are blue by transmitted daylight and reddish violet by transmitted artificial light. A number of cases have been encountered in which the presence of anthoxanthin and other substances have seriously modified the colour reactions and the distribution properties of anthocyanins, occasionally also of the derived anthocyanidins.

Repeated extraction with hot ethyl acetate or ethyl acetate after the addition of sodium chloride (free from magnesium chloride), or amyl alcohol in the case of diglycosides usually served to remove the interfering substances to a sufficient degree. Such processes must be followed by repeated extraction with benzene, or ether and then benzene, or exposure in a vacuum, in order to remove alcohol.

The autumnal colouring of leaves has been examined with the result that cyanidin saccharides are found to preponderate to a very large extent. The extracts were often contaminated with relatively large quantities of tannins and anthoxanthins and it was particularly difficult to determine the glucoside type. Moreover, it appeared that in a large number of the cases, a mixture of anthocyanins based on one anthocyanidin was present. In the presence of so much foreign material a rhamnoglucoside may simulate a diglucoside and a monoglucoside may behave like a rhamnoglucoside. Although the solutions were purified as far as possible, the results in the series of the autumn leaves are not so conclusive in regard to sugar type as those obtained with flower petals. Most of the anthocyanins produced in autumnal colouring are cyanidin 3-saccharides of various types. In this section the material is classified botanically, using Dr Hutchinson's system reversed so that the more highly developed plants are mentioned first in order; the first number indicates the order and the second the family [Hutchinson, 1926].

Summary of results

Flowers		Comments
Abutilon, hybrid, *Firefly*	e	
Agapanthus umbellatus	x	
Allium cyaneum	x	
Anagallis arvensis, scarlet	i	
A. arvensis, blue var.	y	
A. coerulea	z	
A. collina	i	
Anemone coronaria, blue violet var.	x	Partly methylated or mixed with cyanidin derivatives

Flowers		Comments
Anemone coronaria, red violet var.	h	With some delphinidin derivatives. The pigments of the anthers are monoglycosidic or pentoseglycosidic
Aphelandra nitens	i	With some pelargonidin diglycoside
Aubretia deltoidea, var. E. M. Crosfield	e	
Begonia socotrana	h	Acylated, colour reactions before alkaline hydrolysis were correct for 3 : 5-dimonoside
Beloperone guttata	c	In petals and bracts
Billbergia Sanderiana, blue petals	x	With trace of anthocyanin derived from cyanidin
B. Sanderiana, scarlet bracts	A	
Brachycombe iberidifolia	x	Much anthoxanthin
Browallia elata	B	Complex
Brunella grandiflora, violet red var.	e	Complex
B. grandiflora, violet var.	x	Complex
Buddleia Davidii, var. *superba*	C	
B. Davidii, var. *magnifica*	D	
Camellia japonica, var. Lady Clare	a	Very high distribution, almost certainly a cyanidin complex 3-monoglycoside and would doubtless repay detailed investigation. Although the results were not entirely satisfactory, hydrolysis with alkalis gave an anthocyanin of lower distribution and hence the substance is probably an acylated cyanidin glycoside rather than a derivative in which the sugar is attached to the acyl group
C. sasnqua	a	Complex, probably identical with *C. japonica*
Campanula Portenschlagiana	B	
Cistus purpureus	E	Or complex anthocyanin resistant to hydrolysis
Correa speciosa, var. *ventricosa*	c	
Crocus asturicus	x	Kaempferol separated from the acid extracts
C. hadriaticus	x	
C. longiflorus	x	
C. nudiflorus	x	
C. pulchellus	z	
C. sativus	x	With a little complex diglycoside
C. speciosus	z	Extracts contained kaempferol
Dimorphotheca aurantiaca	k	Possibly pentoseglycoside carotenoids are present
Erica hyemalis	a	Much ivory anthoxanthin
Erodium macradenum	x	Methylated. The anthocyanidin is completely extracted by the delphinidin reagent, and not at all by the cyanidin reagent; but it is easily oxidized in strongly alkaline solution. Therefore it may be a new delphinidin derivative, unless a catalyst is present which facilitates the oxidation of malvidin. As this has never previously been observed, it seems more probable that we are dealing here with a new type of delphinidin derivative
E. Reichardii, var. *roseum*	x	
Escallonia edinensis	a	
Eschscholtzia californica, var. *The Mikado*	g	With anthoxanthin
Euphorbia fulgens	a	Much yellow anthoxanthin
Gentiana Lagodechiana	x	
Geranium Endressi	z	
Gilia capitata	x	
Godetia grandiflora, var. *Crimson Glow*	F	
Helianthemum nummularium, a var. with brick red double flowers	a	Much anthoxanthin
Hippeastrum aulicum	j	Co-pigmented
H. equestre	j	
Hydrangea macrophylla vars.	k	After careful purification the reactions and distribution ratios tallied with synthetic delphinidin 3-glucoside. The anthocyanins of red, blue, and violet flowers were found to be identical (correction of Part I)

Flowers		Comments
Hypoestes aristata	e	
Ipomoea Learii	z	
Iris unguicularis	B	
Ixora, hybrid, *Lord Williamson*	g	
Lapageria rosea	a	
Lilium ochraceum	c	
Linaria purpurea	x	
Luculia gratissima	z	Much anthoxanthin
Lychnis viscaria, var. *Blue Bouquet*	B	Indication of partial methylation
Lythrum salicaria, var. *superbum*, pink flowers	z	Strongly co-pigmented
L. salicaria, var. *superbum*, purple flowers	z	Strongly co-pigmented
L. salicaria, var. *Crimson Dwarf*	z	
Macleania punctata	i	
Malvaviscus conjattii	G	
Mesembryanthemum truncatum, var. *roseum*	K	
Monarda didyma, var. *Cambridge Scarlet*	H	Complex
Musa basjoo, bracts	c	
Naegelia cinnabarina	i	
Nemesia strumosa, scarlet var.	a	
Nierembergia coerulea	x	
N. frutescens	x	
Papaver Argemone, spots	g	With a little 3-monoside
P. Argemone, remainder of petals	G	With some pelargonidin 3-saccharide of higher distribution number
P. atlanticum	G	
P. commutatum, spots	g	
P. commutatum, remainder of petals	g	With little of pelargonidin or peonidin derivative
P. somniferum, spots	g	
P. somniferum, remainder of petal	g	As *P. commutatum*
Pelargonium inquinans	i	Noteworthy in a *P.* species
P. saniculaefolium	H	
Penstemon cordifolius	e	With anthoxanthin
Peristrophe speciosa	e	
Phacelia whitlavia	z	
Pisum sativum, purple var.	C	May contain delphinidin but no malvidin derivatives
P. sativum, violet var.	C	With a little malvidin derivative. In both vars. the outer parts of the flower are highly co-pigmented, the inner very little
Platycodon grandiflorum, var. *Mariesii*	x	Much anthoxanthin
Plumbago capensis	h	Much anthoxanthin obscures colour reactions, but the anthocyanin is probably a 3:5-dimonoside
Portulaca grandiflora	K	
Potentilla nepalensis		Acylated cyanidin saccharide. The colour reactions were those of 3-substituted cyanidin derivatives. On hydrolysis an acylated cyanidin was obtained
Pycnostachys Dawei	x	Acylated. Contains a trace of a cyanidin derivative
Raphiolepis Delacourii	a	Much anthoxanthin
Rhodochiton volubile, bracts	f	
Salvia Grahamii	J	Acylated, differing from salvin in that the solution in aqueous Na_2CO_3 is almost pure blue
S. neurepia	J	Acylated. Colour reactions tallied with those of salvin and monardin and differed from those of the anthocyanin of *S. Grahamii*
Silene Schafta	e	
Solanum crispum		Diglycoside of petunidin or delphinidin; the presence of cyanidin is not excluded if an oxidizing catalyst effective in alkaline solution is present
Sollya heterophylla	x	
Sphaeralcea australis	i	
Streptocarpus caulescens	z	Pure, no trace of anthocyanin giving a positive ferric reaction

Flowers		Comments
Streptocarpus Rexii	z	Containing a small amount of cyanidin di-monoside
Suaeda maritima	K	
Tibouchina semidecandra	z	Complex
Tropaeolum majus vars.		The presence of G was confirmed (previously recorded). Deeper coloured vars. contained mixtures in varying proportions of diglycosidic anthocyanins based on cyanidin and delphinidin, ranging from almost pure cyanidin to almost pure delphinidin
Valeriana officinalis, a red var.	c	With a flavone derivative
Veltheimia viridifolia	e	Acylated
Verbena canadensis	e	
V. canadensis, var. *Drummondii*	e	
V. tenera	e	
V. erinoides	B	
V. venosa	B	With small amount of cyanidin diglycoside
V. radicans	B	
V. peruviana	i	
Veronica maritima	x	Complex
Vriesia Duvaliana, petals	x	
V. Duvaliana, bracts	z	Contaminated with a derivative of an anthocyanidin exhibiting a ferric reaction under the usual conditions. The anthocyanin of the bracts could not be extracted even by boiling 1% HCl until the outer skin was removed, when the pigment was yielded to a cold solvent
Zauschneria californica, var. *latifolia*	i	
Zygocactus truncatus	K	
Leaves permanently coloured		
Acalypha macrostachya	a	
Alocasia Lowii	g	Pigmented on underside only
Aphelandra nitens	x	May contain some cyanidin derivative and some monoglycoside. Pigment on underside of leaf only
Calathea insignis	L	
Codiaeum, hybrid, *Emperor Alexander III*	c	
Columnea Schiedeana	h	Only a trace of anthocyanidin was obtained by hydrolysis under the usual conditions, a sparingly soluble mauve precipitate being thrown down. Pigment on underside of leaf only
Cyanotis Kewensis	g	
Hoffmannia Ghiesbreghtii, var. *variegata*	f	
Hoya carnosa, var. *variegata*	c	Pigmented in chlorophyll-free parts only
Iresine Herbstii	K	
I. Lindenii	K	
Perilla nankinensis	e	Complex
Prunus persica, red-leafed var.	a	
Streptocarpus Wendlandii	g	Pigment on underside of leaf only
Strobilanthes Dyerianus	e	
Stromanthe Porteana, var. *variegata*	c	Strongly co-pigmented
Viburnum tomentosum, var. *plicatum*	a	With much anthoxanthin
Other organs		
Cacao beans	a	
Cornus mas (Cornelian cherry)	i	In red berries
Euonymus yedoensis, capsules	a	
Pisum sativum, purple pods	x	
P. sativum, salmon pods	e	
Rubus idaeus, fruits	g	A number of varieties were examined and found to be mainly "*g*" together with smaller amounts of an anthocyanin derived from cyanidin of very high distribution which gave the same colour reactions as cyanidin 3-saccharides

Anthocyanins found in leaves after autumnal coloration

Species and varieties

76–264.	*Elsholtzia Stauntonii*	b
76–263.	*Callicarpa Giraldiana*	e
	C. japonica	e
	C. koreana	e
69–241.	*Ceratostigma plumbaginoides*	b
	Limonium latifolium	b
69–240.	*Steironema ciliatum*	k
68–239.	*Swertia koreana*	b, o
66–233.	*Diervilla Lonicera*	c
	Kolkwitzia amabilis	c
	Viburnum bitchiuense	b
	V. dentatum	b
	V. dilatatum	a
	V. fragrans	a
	V. Lantana	d
	V. Lentago	d
	V. lobophyllum	a
	V. opulus	q
	V. opulus sterile	a, n
	V. orientale	a
	V. Sargentii	f
	V. setigerum	a
	V. theiferum	a
64–229.	*Fraxinus Ornus*	a, n
	Ligustrum obtusifolium	c
60–215.	*Enkianthus campanulatus*	a
	E. deflexus	f
	E. perulatus	b
	Gaultheria procumbens	a
	Leucothoë Keiskei	b
	Oxydendrum arboreum	f, t
	Pieris Mariana	a
	Rhododendron calendulaceum	b
	R. canadense	a
	R. mucronulatum	b
	R. occidentale	b
	R. occidentale exquisita	a
	R. pentaphyllum	b
	R. ponticum	b
	R. reticulatum	a
	R. Schlippenbachii	b
	R. Vaseyi	a, n
	R. yunnanense	a
60–216.	*Gaylussacia baccata*	a
	G. dumosa	a
	Vaccinium caespitosum	b
	V. corymbosum	a
	V. myrtillus	a
	V. ovalifolium	a
	V. pennsylvanicum	b
	V. uliginosum	b
	V. virgatum	b
59–212.	*Aralia chinensis Mandschurica*	h, u
59–209.	*Cornus alba*	b
	C. alba Spaethii	h
	C. Amomum	f
	C. Baileyi	c
	C. florida	b
	C. glabrata	b
	C. Koenigii	a
	C. Kousa	a
	C. Nuttallii	c
	C. obliqua	c
	C. Walteri	a
59–211.	*Nyssa sylvatica*	a, n
57–200.	*Acer circinatum*	a
	A. dasycarpum	a
	A. Davidii	c
	A. Ginnala	b
	A. griseum	c
	A. palmatum	a
	A. pseudoplatanus	b
	A. rubrum	a
	A. rufinerve	a
	A. Tschonoskii	d
	A. palmatum dissectum	b
	A. palmatum septemlobum	b
	A. palmatum septemlobum elegans	a
	A. palmatum roseo-marginatum	b
	A. platanoides Reitenbachii	d
57–205.	*Rhus canadensis*	b
	R. cotinoides	a
	R. cotinus	a
	R. cotinus atropurpurea	b
	R. glabra laciniata	b
	R. Potaninii	b
	R. toxicodendron	k
	R. trichocarpa	d
	R. typhina	a
	R. typhina laciniata	a
	R. verniciflua	a
55–194.	*Aegle sepiaria*	g
54–193.	*Vitis Coignetiae*	a
	V. Englemannii	b
	V. Henryana	a
	V. inconstans	a
	V. pulchra	e
	V. quinquefolia, var. *Engelmannii*	a, n
51–173.	*Euonymus alata*	b
	E. americanus	a
	E. Bungeanus	a
	E. europaeus	a
	E. Maackii	a
	E. nikoensis	b
	E. oxyphyllus	b
	E. planipes	a, n
	E. radicans	b
	E. sanguineus	a
	E. verrucosus	b, n
	E. Wilsonii	b, n
	E. yedoensis	b
48–162.	*Carpinus caroliniana*	b
	Corylus americana	b
48–163.	*Quercus alba*	b
	Q. coccinea	f
	Q. coccinea splendens	b
	Q. glandulifera	a
42–151.	*Corylopsis yunnanensis*	e
	Disanthus cercidifolius	a
	Fothergilla Gardenii	h
	F. monticola	g
	Hamamelis japonica flavopurpurascens	v
	H. japonica Zuccariniana	w
	Liquidambar styraciflua	a
	Parrotia persica	l
40–143.	*Amelanchier canadensis*	b
	A. florida	b
	A. oblongifolia micropetala	a

40–143.	*Aronia arbutifolia*	a		*Sorbus Folgneri*	d
	A. arbutifolia macrocarpa	d		*S. hupehensis*	a
	A. melanocarpa	a		*S. Sargentiana*	a
	A. melanocarpa elata	b		*S. serotina*	d
	Cotoneaster acutifolia	b		*Spiraea japonica ruberrima*	a
	C. Franchetii	b		*S. nipponica*	d, p
	C. frigida	a		*S. Sargentiana*	a, n
	C. Henryana	b		*Stephanandra incisa*	a
	C. horizontalis	a		*Stranvaesia salicifolia*	i
	C. rotundifolia	a		*S. undulata*	a
	Crataegus acutiloba	b	39–139.	*Itea virginica*	a
	C. Boyntoni	b, r	39–141.	*Ribes americanum*	j
	C. collina	b		*R. cynosbati*	b
	C. Downingii	d, r		*R. odoratum*	a
	C. opposita	a	39–142.	*Deutzia gracilis carminea*	d
	C. prunifolia	a		*D. scabra*	h
	Fragaria indica	a		*Hydrangea quercifolia*	c
	Malus coronaria elongata	b	34–124.	*Eucryphia glutinosa*	a
	M. ioensis plena	b	34–123.	*Hypericum patulum*	b
	M. Tschonoskii	b		*H. patulum Forrestii*	b
	Neillia longiracemosa	d	32–108.	*Stewartia pentagyna*	b, o
	Nuttallia cerasiformis	b		*S. Pseudo-Camellia*	b, o
	Photinia serrulata	a	21–75.	*Punica Granatum*	a
	Prunus Davidiana	a	14–47.	*Bergenia cordifolia*	a
	P. glandulosa albo-plena	a	5–19.	*Berberis acuminata*	b, o
	P. incisa serrata	b		*B. aemulans*	a
	P. pennsylvanica	h		*B. angulosa*	a
	P. Sargentii	a		*B. Beaneana*	a
	P. Sieboldii	b, s		*B. consimilis*	b
	P. subhirtella	a		*B. dictyophylla*	a
	P. thibetica	b		*B. Ferdinandi-Coburgii*	f
	P. yedoensis	d, o		*B. Jamesiana*	a
	Pyrus Calleryana	a		*B. Julianae*	a
	P. Pashia	a		*B. replicata*	b
	Rosa blanda	a		*B. Sargentiana*	b
	R. nitida	a		*B. Thunbergii*	b
	R. rugosa	b		*B. Tischleri*	b
	R. smoleschina	b		*B. Veitchii*	d
	R. spinosissima	a		*B. virescens*	a
	R. virginiana	b		*B. yunnanensis*	b
	Rubus allegheniensis	a		*Mahonia japonica*	b
	R. chroösepalus	b		*Podophyllum Emodi*	a
	R. cinnamomeus	a	1–7.	*Cercidiphyllum japonicum*	h

Species hybrids

60–215.	*Rhododendron altaclarense*	b	48–163.	*Quercus Leana* (=*Q. imbricaria* × *velutina*)	f
	R. viscosepala	b			
	R. occidentale × *calendulaceum*	b	40–143.	*Rosa calocarpa* (=*R. rugosa* + *indica*)	a
	R. malvatica × *Kaempferii* "Jeannette"	b			

Horticultural forms of unknown origin

60–215.	*Azalea*: "Raphael de Smet"	a		*A. japonicum aureum filicifolium*	b
	"Corneille"	a		*A. palmatum reticulatum*	a
	"Il Tasso"	a		*A. palmatum scolopendrifolium septemlobum Osakozuki*	b
	"Joseph Baumann"	a			
	KH 101 "Mrs A. Waterer"	a		*A. palmatum scolopendrifolium versicolor*	b
	KH 177 "Whitethroat"	a			
	KH 182	a		*A. platanoides laciniatum*	b
57–200.	*Acer Davidii Forrestii*	b	40–143.	*Pyrus communis*	c

a Cyanidin 3-monoside.
b Cyanidin monoside.
c Cyanidin 3-pentoseglycoside.
d Cyanidin pentoseglycoside.
e Cyanidin 3 : 5-dimonoside.
f Mixed cyanidin saccharides, probably chiefly 3-saccharides.
g Cyanidin 3-bioside.

h Cyanidin diglycoside.
i Pelargonidin 3-monoside
j Pelargonidin 3-pentoseglycoside.
k Delphinidin 3-monoside.
l Delphinidin 3 : 5-dimonoside.
n These all contained amounts of diglycoside as well as the main anthocyanin.
o The distribution of the anthocyanins in these cases was intermediate between monoside and pentoseglycoside.
p The anthocyanin is largely pentoseglycoside, but contains a small amount of diglycoside. The aglycone from the pentoseglycoside fraction consists of cyanidin together with some anthocyanidin, in lesser amount, with a high distribution to the cyanidin reagent.
q Largely cyanidin monoside containing some diglycoside which on acid hydrolysis gave a mixture of cyanidin and some anthocyanidin based on delphinidin.
r The anthocyanins were monosides and the aglycones gave reactions indicating mixtures of cyanidin with peonidin. Cyanidin was certainly present, but the constituent with a high distribution to the cyanidin reagent may have been pelargonidin. Peonidin was considered more likely on account of marked blueing on dilution with absolute ethyl alcohol.
s These two resemble *Spiraea nipponica* in that the aglycone consists of cyanidin containing some anthocyanidin with a high distribution to cyclohexanol-toluene. It may have been an acylated cyanidin, since unlike *Crataegus Boyntonii* and *C. Downingii*, after repeated washing of a cyclohexanol-toluene extract, the ferric reaction was still completely positive.
t Mixture of cyanidin monoside with a cyanidin 3-saccharide of higher distribution.
u The anthocyanidin was cyanidin, but the saccharide was of unusual type. The distribution (without salt) was nil, but on saturating with salt the pigment was partly extracted. This behaviour remained unaltered after purification (by repeated extraction with amyl alcohol and returning to 1% HCl and repeating the process). The colour reactions were dirty, but indicated a 3-saccharide.
v Diglycosidic anthocyanins based mainly on malvidin together with a little cyanidin.
w Similar to the above variety *flavo-purpurascens*, except that the amount of cyanidin present is greater.
x Delphinidin diglycoside.
y Malvidin 3-monoside.
z Malvidin 3 : 5-dimonoside.
A Pelargonidin 3 : 5-dimonoside and a cyanidin diglycoside in more or less equal proportions.
B Delphinidin 3 : 5-dimonoside.
C Petunidin diglycoside.
D Petunidin 3-pentoseglycoside.
E Petunidin 3-monoside.
F Peonidin diglycoside.
G Pelargonidin 3-bioside.
H Pelargonidin 3 : 5-dimonoside.
J Pelargonidin diglycoside.
K Betanin type of pigment.
L Delphinidin 3-pentoseglycoside.

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CCXVI. SPECTROGRAPHIC STUDIES ON THE ANTIMONY TRICHLORIDE REACTION FOR VITAMIN A

III. THE RELATION OF THE SPECTRAL ABSORPTION OF THE BLUE SOLUTIONS OF OILS TO THAT OF THEIR CONCENTRATES

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Measurements of blue solutions of oils and their corresponding concentrates have been made by a number of authors using the tintometer [Coward *et al.* 1931; Dyer, 1933; Crews & Cox, 1934; Notevarp, 1935; Morgan & Pritchard, 1935], and also by some who have examined them both spectrographically and with the tintometer [Morgan *et al.* 1935].

Values for the ratio between the blue value of oils and that of their concentrates have been found between 1·16 (minimum found by Dyer [1933]) and 6·35 (maximum found by Morgan & Pritchard [1935]).

As calculated by means of the equations from Part I of these studies [Notevarp & Weedon, 1936], this corresponds to a minimum ratio between the 603 and 618 mμ bands of 0·65 and a maximum of 3·6. The ratios between the actually measured values for the two bands of Morgan *et al.* [1935] lie between 1·0 and 1·75.

We have shown in Part I that for purely physical reasons a concentrate gives a linear blue value[1] approaching twice that of an oil with the same absorption value. Higher or lower blue value ratios than a little under 2:1 must therefore signify that the absorptions at 603 mμ (oils) and at 618 mμ (concentrates) are not equal.

Higher blue value ratios are explained by inhibition of the reaction with the oil by the saponifiable fraction. In Part II of these studies [Notevarp & Weedon, 1938] this inhibition was shown to be considerable in all cases when the reaction was carried out in the usual way.

Loss of chromogen is the only reasonable explanation for 618 mμ values lower than the corresponding 603 mμ values. It follows therefore that as all hitherto published results for oils must presumably have been carried out under conditions favouring inhibition, all concentrate/oil blue value ratios as low as 1·0 or less show that substantial losses of chromogen must have occurred. Values above 2 or more indicate strong inhibition of the type found in unoxidized oils.

The object of this study was to investigate whether there exists a definite relation between the blue value and absorption of oils and concentrates.

[1] To avoid misunderstanding "linear blue value" is defined as the value obtained by direct calculation to 18·18 g./l. from the concentration which gives a blue value of 6·0.

Experimental

The relation between oil and concentrate absorption determined with ordinary reagent

We measured $E_{603/618m\mu}$, blue value and $E_{328m\mu}$ for a number of oils both fresh and after some oxidation, and for their concentrates. The results are collected in Table I.

Table I

				Linear B.V.			$E_{328m\mu}$		
Oil no.	$E_{603m\mu}$	$E_{618m\mu}$	Ratio	Oil	Conc.	Ratio	Oil	Conc.	Ratio
15	0·21	0·26	1·24	2·5	4·9	1·96	0·19	0·10	0·53
3 unoxidized	0·54	0·94	1·74	5·9	16·0	2·70	0·48	0·40	0·84
3 max. by oxidation with air	0·90	0·94	1·05	9·6	16·0	1·67	0·48	0·40	0·84
2*a*	0·30	0·82	2·70	2·3	15·2	6·60	0·38	0·32	0·84
2*b*	0·70	0·82	1·17	7·8	15·2	1·95	0·38	0·32	0·84
4*a*	0·49	0·95	1·94	5·7	18·8	3·30	0·45	0·37	0·82
4*b*	0·85	0·95	1·12	9·8	18·8	1·92	0·45	0·37	0·82
1*a*	0·36	1·05	2·90	6·0	24·0	4·00	0·52	0·45	0·87
1*c*	0·88	1·05	1·19	10·4	24·0	2·30	0·52	0·45	0·87
7	1·80	2·35	1·30	16·0	30·0	1·88	0·90	0·77	0·86
18	17·8*	17·6	0·99	320·0	250·0	1·09	7·40	6·10	0·83

* Max. at 610mμ.

The results confirm the deductions made in the introduction. After maximum absorption obtainable by oxidation with air was reached the $E_{618}/E_{603m\mu}$ ratio varied from 1·05 to 1·30. Unoxidized oils gave ratios from 1·74 to 2·9. One potent oil gave 0·99, probably because there was much less saponifiable matter in relation to the amount of chromogen, as the ultra-violet absorption showed that losses during preparation had not been excessive.

For reasons already explained, the ratio between the blue values was higher, ranging from 1·67 to 2·3, in the oils where maximum obtainable absorption had been reached. For unoxidized oils the range was 2·7 to 6·6. Oil No. 18 was an exception, as the properties of this oil approached those of a concentrate.

The figures are by no means unique, and are included merely to demonstrate that the relation between the blue values and 603–618mμ absorption of oils and their concentrates varies considerably, not only in the case of unoxidized oils, but also for oils in which maximum absorption obtainable with air had presumably been reached. Apart from one oil of exceptionally low potency, the ratio between the ultra-violet absorption of the oils and the concentrates is constant within the limits of error of the method of determination and is in close agreement with the ratio demonstrated earlier by one of us [Notevarp, 1935]. The small loss of chromogen during preparation of the unsaponifiable fraction should therefore be approximately constant.

From these facts it must be concluded that there is no constant relation between the blue values, measured either optically or spectrographically, of oils and those of their concentrates when the ordinary reagent is used. For fresh oils strong inhibition makes the values much too low; the inhibition can be substantially reduced by oxidation with air, but even when maximum values have been reached by this method the fact that the concentrate values are definitely higher than the oil values shows that inhibition still exists, while the variations in the ratios demonstrate that no well-defined part of the inhibition is removed.

If, therefore, the 603/618 mμ absorption is caused by vitamin A, we have further evidence of the view put forward in Part II of these studies, that the blue value as usually determined is not a true indication of the vitamin A potency of cod liver oils, even when the facts set out in Part I of these studies are taken into consideration.

If maximum absorption resulting from oxidation by air has been reached, however, and in Part II of these studies it was shown that this is probable in a majority of cases, the error is not much greater than the inherent inaccuracies of the blue value determination, a further explanation of the comparatively reliable blue values obtained in routine assays.

The relation between oil and concentrate absorptions using oxidizing reagent

Oils of low potency. $E_{603m\mu}$ and $E_{618m\mu}$ and the corresponding blue values were determined for cod liver oils using the oxidizing reagent containing 0·1 g./l. bromine described in Part II of these studies.

The oils were in various stages of oxidation and ranged from quite fresh ones to slightly rancid ones (oil 15). The results are recorded in Table II.

Table II

Oil no.	$E_{603m\mu}$	$E_{618m\mu}$	Ratio	Linear B.V. Oil	Linear B.V. Conc.	Ratio	$E_{328m\mu}$ Oil	$E_{328m\mu}$ Conc.	Ratio
15	0·32	0·26	0·82	2·9	4·9	1·69	0·19	0·10	0·53
2	0·94	0·82	0·87	11·0	17·5	1·59	0·38	0·32	0·84
19	1·06	1·04	0·98	12·4	20·2	1·63	0·40	0·36	0·90
1*b*	1·32	1·15	0·85	15·0	24·2	1·61	0·52	0·45	0·86
20	1·60	1·52	0·95	21·0	35·0	1·66	0·61	0·56	0·92
21	1·80	1·65	0·92	24·2	39·0	1·62	0·72	0·64	0·89
Average ratio			0·90			1·63			0·88

The average ratio between $E_{328m\mu}$ for oils and $E_{328m\mu}$ for concentrates was about the same as the average ratio between $E_{603m\mu}$ and $E_{618m\mu}$, with about the same deviations from the average in the two cases. (Oil No. 15 was not included in the average for ultra-violet absorption ratio, as there was reason to believe that the value obtained for this oil was too high owing to the presence of substances other than vitamin A). The blue value ratio, as would be expected, was higher (average 1·63), with about the same deviations as the other ratios. This shows that when the bromine reagent is used there is a definite relation between the $SbCl_3$ reaction values for oils and their concentrates, corresponding to the well known relation between the ultra-violet absorption values. The effect of preparation of the concentrates is a loss in $E_{603/618m\mu}$ of about 10%, together with a shifting of the band; these two effects together determine the blue value ratio, according to the relations discussed in Part I of these studies.

Oils of potency above the average. Three cod liver oils of potency considerably above the average were measured by means of the bromine reagent. Unfortunately no fresh samples were available, as cod liver oils of such potency are scarce and are only available when oils from single livers are prepared. The results are recorded in Table III.

The first two oils showed lower $E_{603/606m\mu}$ than E_{618} values, indicating that the inhibition by the rancid oil was not completely removed. The most potent oil showed the usual relation, while the blue value ratio was considerably lower than

for less potent oils. This is because the absorption band was no longer at 603 mμ, but had moved to 606 mμ, indicating that the properties of the oil were approaching those of a concentrate.

Table III

Oil no.	$E^{608\,m\mu}_{606\,m\mu}$	$E_{618\,m\mu}$	Ratio	Linear B.V. Oil	Linear B.V. Conc.	Ratio	$E_{328\,m\mu}$ Oil	$E_{328\,m\mu}$ Conc.	Ratio
17	4·5	5·3	1·17	51	84	1·64	2·0	1·7	0·85
16	13·4	15·2	1·13	190	296	1·55	5·5	4·7	0·85
18	19·0	17·6	0·93	300	350	1·17	7·4	6·1	0·83

Shifting of the band from 603 to 618 mμ

No experimentally proved explanation of the shifting of the main absorption band of the $SbCl_3$ reaction from 603 to 618 mμ has come to our notice. The occurrence of vitamin A in an esterified form in oils, and as a free alcohol in concentrates, may be an explanation, while we ourselves have ascribed the

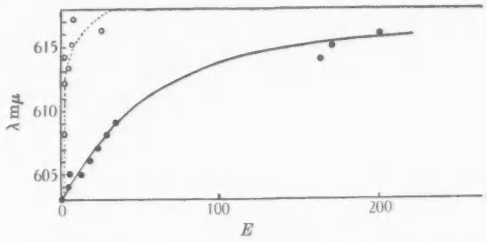

Fig. 1. Alteration in wave-length with increasing $E^{10g/l}$.

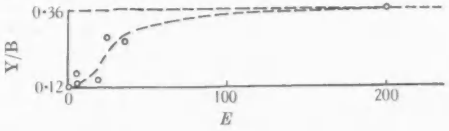

Fig. 2. Alteration in Y/B ratio with increasing $E^{10g/l}$.

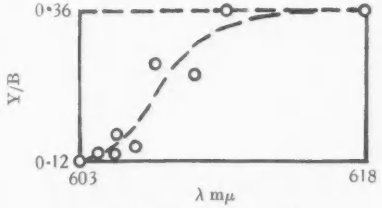

Fig. 3. Alteration of Y/B ratio with increasing wave-length.

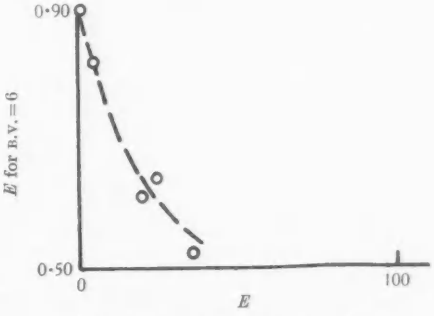

Fig. 4. Alteration of E for B.V. = 6 with increasing $E^{10g/l}$.

shifting to the change in the solvent effected by the unsaturated glycerides of the oils [Notevarp & Weedon, 1936]. If the latter explanation is correct, the maximum should move gradually towards 618 mμ with rising E values.

Table IV

Oil no.	No. times fortified	E_{max}	mμ	Ratio Y/B	$E_{B.V.=1}$	$E_{B.V.=6}$
15	—	0·32	603	0·12	6·5	0·90
15	21	5·30	605	0·16	6·8	0·84
15	101	24·00	607	0·27	8·6	0·64
17	—	4·50	604	0·13	—	—
17	41	200·00	616	0·36	—	—
16	—	13·40	605	0·12	7·2	0·80
16	11	163·00	614	—	—	—
18	—	19·00	606	0·14	8·9	0·61
18	2	35·00	609	0·26	10·0	0·52
14 (halibut)	—	170·00	615	—	—	—

As an experimentally proved explanation would seem to be of interest, both theoretically and as a basis for a better understanding of the relation between the blue value and chromogen content of oils, we have measured absorption, location of bands and blue values of oils and of the same oils fortified with their own concentrates, using the bromine reagent. We have also measured the ratio of yellow to blue matching in the Tintometer, as this indicates the location of the band, while on the other hand the relation between this ratio and the location of the band is unknown.

The results have been collected in Table IV together with the E values corresponding to a blue reading of 6, shown graphically in Figs. 1 and 2, 3 and 4. In Fig. 1 are also shown some values obtained by using the ordinary reagent.

Discussion

Comparison of the three methods for the determination of vitamin A in oils and their concentrates, i.e. blue value, absorption of $SbCl_3$ blue solution and ultra-violet absorption, shows that with the ordinary reagent there is in all cases definite inhibition of the $SbCl_3$ reaction with oils, making absorption values and consequently blue values too low. The degree of inhibition varies for different oils, even when the major portion of the inhibiting factors has been removed by oxidation with air. This suggests strongly that although the blue value of oils obtained by the use of ordinary reagent in a majority of cases gives a good indication of the vitamin potency, the varying degree of inhibition is a source of error which cannot be controlled when such reagent is used, and may, in the case of fresh oils, lead to very considerable errors.

With the use of the bromine reagent the relation between the absorption of $SbCl_3$ blue solutions of oils and their concentrates is constant within the limits of error of the determinations for normal oils of low to medium potency and is the same as the corresponding relation between the ultra-violet absorptions. For such oils, therefore, the inhibition of the 603 mμ band is practically completely removed, consequently the ratio between the blue values is constant and about 1·6, in accordance with the conclusions reached in Part I of these studies.

The values for three oils of higher potency do not allow a very definite interpretation, probably because the oils were of uneven freshness. A considerable portion of the inhibition must have been removed, but although $E_{603\,m\mu}/E_{618\,m\mu}$

is still above 1 for two of the oils, the B.V. ratio is about the same as for ordinary oils with an E ratio of about 0·9. Complete removal of the inhibition would therefore have resulted in a lower B.V. ratio indicating shifting of the bands towards 618 mμ. This is in fact the case with the third oil, where the E ratio is normal.

Table IV and Figs. 1–4 throw light on the mechanism of the shifting of the band from 603–618 mμ. A greater number of determinations would have been desirable, especially as the accuracy of determination of both wave-length of the maximum absorption and $Y:B$ ratio is not very great. Nevertheless, the values shown demonstrate that there is a continuous change in both wave-length and $Y:B$ ratio with rising E values.

The shifting of the band is therefore due to some gradually changing influence, most probably the concentration of the glycerides, as we have suggested earlier. There should therefore exist a simple mathematical relation between the distance the band has moved, $\Delta\lambda$ (in mμ), and the concentration of glycerides, which again is inversely proportional to the chromogen concentration, i.e. to E. Such a relation would be of a tangential type. In Fig. 1 the curve corresponds to a function

$$E = 50tg\,6\Delta\lambda$$

where $6\Delta\lambda$ is expressed as degrees. As $\Delta\lambda$ can vary from 0 to 15, the function varies from 0 to ∞. It will be seen that the measured values are grouped sufficiently close around the curve to make the simple tangential relation highly probable.

Although the three other figures contain only a small number of values, they nevertheless show that for E values somewhere about 50, or, according to Fig. 1, for maximum wave-lengths above 610 mμ, the Y/B ratio and the $E_{\text{B.V.}-6}E$ ratio approach those of a concentrate. This means that if insignificant inhibition of the main band is assumed, linear blue values above 5–600 should be proportionate to the content of chromogen, as is the case with concentrates.

The curve obtained with the ordinary reagent in Fig. 1 indicates that the shifting of the band is more rapid than when the bromine reagent is used. On the other hand, the ordinary reagent involves the inhibition which is removed by the bromine; the picture is thus complicated and it would seem of little interest to study this relation closer.

Summary

By measurements of the $SbCl_3$ absorption of oils and their concentrates with the ordinary non-oxidizing reagent, no definite relation was found between oil and concentrate absorption and blue values, even when maximum absorption of the oils by oxidation with air had been reached. The oil absorption values were lower than those of the corresponding concentrates, indicating that there remained inhibiting factors in varying amounts.

When the bromine reagent was used the relation between oil absorption and concentrate absorption values was constant for normal oils, and corresponded closely to the relation between the ultra-violet absorption values. The blue value ratio was also constant, but in accordance with the facts set out in Part I of these studies the blue values of the concentrates were higher than those of the oils.

Bromine reagent would therefore appear to eliminate the difference between the $SbCl_3$-reaction with oils and their concentrates except the shifting of the absorption maximum. If the laws governing the latter are known, there should be no reason to isolate the unsaponifiable matter to obtain a reliable blue value for normal oils.

Measured values for the relation between wave-length and E indicated that there is a continuous movement of the band from 603 mμ towards 618 mμ with increasing E. If the shifting is caused by the concentration of glycerides the shifting should follow a tangential function; this is shown to be probable. The ratios between yellow and blue matching and the E value for a blue reading equal to 6 probably also change continuously. The curves found for all relations show that the properties of a concentrate are approached from about $E=50$; above this value, therefore, the linear blue values should be proportionate to the chromogen content.

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CCXVII. THE PROTEOLYTIC ENZYMES OF SPROUTED WHEAT. III

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In two previous communications the author [1936, 1, 2] has described the chief properties of the proteinase and dipeptidase found in aqueous extracts of sprouted wheat. The present paper is a continuation of these studies and deals primarily with the action of the proteinase on gelatin, ovalbumin and on the principal proteins of wheat, namely, gliadin and glutenin, both separately and in the form of gluten.

Experimental

The Sørensen method of formaldehyde titration in two stages, (*a*) and (*b*), again formed the basis of investigation, enzyme activity being recorded in terms of *N*/20 NaOH increase.

Substrates. The gluten, gliadin and glutenin used as substrates were prepared from a sample of untreated Canadian flour. The gluten was obtained from a dough made by mixing 100 g. flour with 55 ml. tap water. The dough was first immersed in tap water at 18° for 1 hr. and was then kneaded under a stream of tap water until all starch, etc. had been removed. After a final washing in distilled water and the removal of surplus water the gluten mass was cut into small pieces and mixed with sufficient *N*/5 acetic acid to yield a final gluten concentration of 10%, allowance being made for the water retained by the gluten. This water content was determined by drying a sample for 24 hr. at 100°. Gluten, of course, consists almost entirely of gliadin, glutenin and water, the two proteins together forming about 34% and the water about 66% of the mass, although small amounts of lipins, mineral matter etc., remain firmly adherent.

The gliadin and glutenin were prepared simultaneously by a modification of methods proposed by Troensegaard [1931] for gliadin and by Damodaran [1931] for glutenin. Wet gluten, obtained as described above, was cut into small pieces and the gliadin extracted by several changes of 70% ethyl alcohol at 28°. The dissolved protein was precipitated by concentration of its solution under reduced pressure at 30° and purified, firstly by treatment with ether to remove fats, lipins etc., secondly with distilled water to remove mineral matter etc., and finally by re-solution in 70% alcohol and precipitation by concentration again. Dehydration with alcohol in gradually increasing concentration up to absolute alcohol completed the process.

Treatment of the air-dried residue from the gliadin extraction with 0·2% NaOH yielded a solution of glutenin which was precipitated by adjusting the *p*H to 6·8–7·0 (the isoelectric point of glutenin) by addition of acetic acid. The precipitate was purified by repeated dissolution in 0·2% NaOH and subsequent precipitation with 0·4% acetic acid at *p*H 6·8–7·0. The protein precipitate was washed with distilled water by decantation until free from acid, then with absolute alcohol and finally with ether. Both preparations were thereafter kept *in vacuo* over P_2O_5.

Results

Determination of pH optimum for gelatin. A freshly prepared 10 % aqueous solution of Gold Leaf gelatin was used as substrate. The corrected curve shown in Fig. 1 has an optimum at *p*H 5·1. In the same figure are included the experimental test and control curves from which the corrected curve was calculated by difference. The control curve was determined on mixtures containing buffers and enzyme extract only.

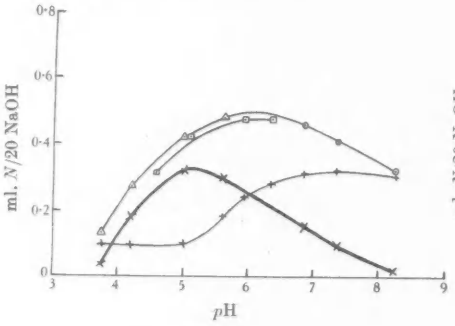

Fig. 1.

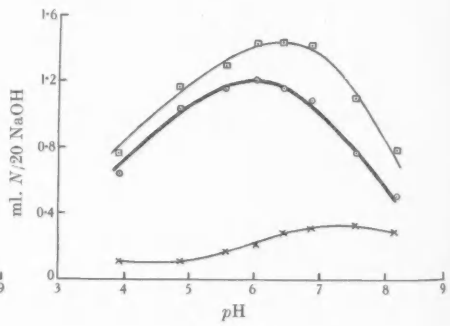

Fig. 2.

Fig. 1. *Effect of variation in* p*H on gelatin hydrolysis.* Conc. of gelatin in reaction mixtures: 2·0 %. Enzyme dilution 2 : 7. *M*/7 acetate, *M*/7 KH_2PO_4-NaOH and *M*/7 K_2HPO_4-NaOH buffers. Titres as ml. *N*/20 NaOH per 5 ml. sample. Reaction 4 hr. at 40°. ◭ Acetate buffers. ⊡ KH_2PO_4-NaOH buffers. ⊙ K_2HPO_4-NaOH. + Control curve. × Corrected curve.

Fig. 2. *Effect of variation in pH on gluten hydrolysis.* Conc. of gluten in reaction mixture: 3·0 %. Enzyme dilution 2 : 7. *M*/7 potassium phosphate-NaOH buffers. ⊡ Test curve. ⊙ Corrected curve. × Control curve.

Action of proteinase on ovalbumin. Over the range covered by acetic acid—sodium acetate buffers (*p*H 3·75–5·7) no increase in titre above that of the control value was observed after 4 hr. at 40°. This result was confirmed after 4 hr. and 24 hr. respectively, at 40° by Harris's [1923] method of titration in 85 % alcohol using thymolphthalein as indicator. As substrate, a filtered 5 % solution of dried egg albumin (Merck) in *N*/5 acetic acid was used.

Determination of pH optimum for gluten. The effect of variation in *p*H on gluten hydrolysis by wheat proteinase was determined after 4 hr. at 40°. The results in Fig. 2 show that the corrected curve has an optimum range of *p*H 5·6–6·4, with a mean value of *p*H 6. All reaction mixtures contained a considerable amount of precipitated gluten proteins.

Effect of NaCl on gluten hydrolysis. In view of the inhibitory effect of certain concentrations of NaCl on edestin hydrolysis by wheat proteinase [Mounfield, 1936, 1, Fig. 7], parallel experiments were conducted at *p*H 6 with gluten (final conc. 2·4 %) as substrate. With increasing concentrations of NaCl up to 0·25 *M* the difference from the control value without salt did not exceed 0·04 ml. *N*/20 NaOH which is below the limit of experimental error. As in the preceding experiment all reaction mixtures contained a curdy white precipitate of gluten proteins, the amount of which was not, however, increased by increasing salt concentration. Sodium chloride up to 0·25 *M* is therefore without influence on gluten hydrolysis by wheat proteinase.

Relation between initial reaction velocity and gluten concentration. Variation in gluten concentration produced the results indicated by the upper and lower lines in Fig. 3. The increases for total titre ($a+b$) and titre (b) did not bear a constant ratio to each other as expected and both are therefore reproduced in the figure after deduction of control values without gluten. With increased concentration of gluten there was a corresponding increase in the amount of precipitate in the reaction mixtures.

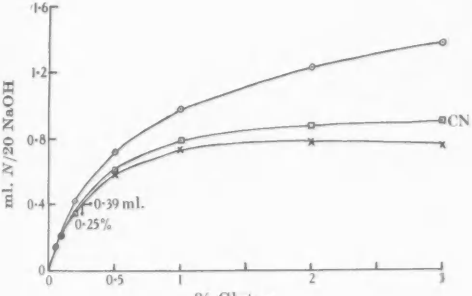

Fig. 3.

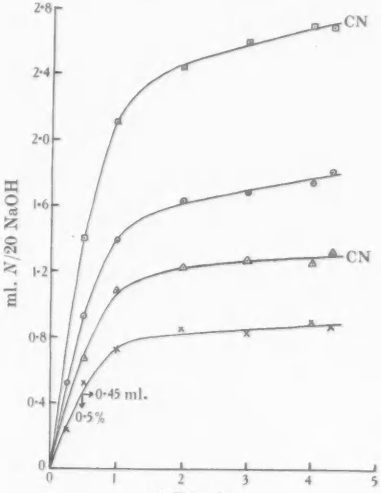

Fig. 4.

Fig. 3. *Effect of gluten concentration.* Enzyme dilution 2 : 7. $M/7$ potassium phosphate-NaOH buffer at pH 6. Reaction 4 hr. at 40°. ⊙ Total titre without cyanide. × Titre (b) without cyanide. ⊡ Titre (b) with cyanide.

Fig. 4. *Effect of edestin concentration.* Enzyme dilution 2 : 7. $M/7$ acetate buffers at pH 4·1. ⊙ Total titre without cyanide. × Titre (b) without cyanide. ⊡ Total titre with cyanide. ⊿ Titre (b) with cyanide.

The curve for titre (b) reaches a maximum at a gluten concentration of approximately 1·5 % whereas the curve for total titre continues to rise over the whole range of substrate concentration examined.

Relation between initial reaction velocity and edestin concentration. The effect on proteinase activity of variation in edestin concentration differs to some extent from that obtained with gluten as substrate. The ratio between the increases in total titre and titre (b) (see Fig. 4), is almost constant throughout at 2 : 1.

Effect of NaCN on hydrolysis of edestin at varying concentrations of the latter. Increases in titre in the presence of cyanide may conceivably be due to a more complete hydrolysis of the cleavage products rather than to a simple acceleration of reaction velocity. Data which appear to throw some light upon this problem were obtained by redetermining the curves showing the effect of edestin concentration on proteinase activity in the presence of a constant concentration of cyanide, i.e. 0·01 M (see Fig. 4). The curves for total titre and for titre (b) in the presence of cyanide are proportionately higher in both cases than are the corresponding curves in the absence of cyanide.

Influence of cyanide upon proteinase activity using gluten as substrate. The addition of cyanide to mixtures containing gluten as substrate caused no activation of the wheat proteinase as measured by increase in total titre. There was, however, some alteration in the ratio of titre (*a*) to titre (*b*): the latter was increased slightly at the expense of the former, total titre remaining the same as in the absence of cyanide. The middle line in Fig. 3 represents the effect upon titre (*b*) of a constant concentration (0·01 *M*) of cyanide when the concentration of gluten is varied.

Effect of cyanide on control values. During the determination of control values (without added substrate) in connexion with the foregoing cyanide experiments it became evident that at certain *p*H values the cyanide caused not only a small but definite increase in total titre over and above the corresponding control value without cyanide but also an appreciable alteration in the ratio titre (*a*) to titre (*b*). The normal control mixtures consisted of enzyme extract + buffer, with water in place of substrate solution: the cyanide controls contained NaCN to a final concentration of 0·01 *M*. In Table I are presented a few typical values for titres (*a*) and (*b*) at different *p*H values with and without cyanide.

Table I

*p*H value		4·11	5·23	6·02	7·15	8·08
Without cyanide	Titre (*a*)	0·02	0·02	0·01	0·02	0·02
	Titre (*b*)	0·08	0·10	0·20	0·29	0·27
	Total	0·10	0·12	0·21	0·31	0·29
With cyanide	Titre (*a*)	0·00	0·00	−0·04	−0·08	−0·09
	Titre (*b*)	0·11	0·14	0·28	0·44	0·46
	Total	0·11	0·14	0·24	0·36	0·35

The influence of cyanide is first to produce a slight increase in total titre and secondly to enlarge titre (*b*) at the expense of titre (*a*). Both these changes are more pronounced at the more alkaline reactions.

Action of wheat proteinase on gliadin and glutenin. Gliadin and glutenin prepared in the manner already outlined do not appear to be affected to any great extent by wheat proteinase. Over the range *p*H 3·75–8·2 no increases in titre greater than those of the control could be detected except at about *p*H 5. At this point a slight optimum was discernible in the case of gliadin. Substrate concentrations of 2·0 % in *N*/5 acetic acid were employed. Doubling the concentration of protein produced no further effect upon the titre increase. These results were confirmed on four occasions with separate preparations of gliadin and glutenin.

In view of any possible modification of the glutenin by the NaOH used in its preparation the action of wheat proteinase was determined on a sample of glutenin prepared as already described with the exception that solution in 0·2 % NaOH and precipitation with 0·4 % acetic acid were omitted, the air-dried residue being simply dehydrated with increasing concentrations of alcohol. The proteinase was found capable of attacking this substrate and although the extent of scission was by no means as great as in the case of gluten it was sufficiently great to reveal a slight optimum at approximately *p*H 5·5.

Discussion

The investigation of the effect of wheat proteinase on proteins other than edestin reveals a somewhat unexpected range of specificity. Gelatin and gluten are readily attacked whereas ovalbumin and the constituent proteins of gluten—

gliadin and glutenin—are not. Gelatin shows an optimum at *p*H 5·1 (see Fig. 1), a value which is close to the isoelectric point of the protein, *p*H 4·9 [Pauli & Modern, 1925]. Papain [Willstätter *et al.* 1926], yeast proteinase [Willstätter & Grassmann, 1926] and malt proteinase [Lüers & Malsch, 1929] also attack gelatin optimally at *p*H 5. These facts suggest that plant proteases, generally, hydrolyse the unionized form of gelatin.

It will be observed from Fig. 1 that phosphate used as buffer has a slight retarding influence on the rate of proteolysis. The phosphate curve is, however, parallel over its whole range with the acetate one. The importance of the control curve correction is made particularly manifest in Fig. 1, an apparent optimum of *p*H 6 being reduced thereby to one of *p*H 5·1. It should be borne in mind, however, that the use of the correction curve (obtained from mixtures of buffer and fresh enzyme extract only) is based on the assumption that on the addition to the control mixture of another substrate such as gelatin the latter will not be preferentially attacked by the proteinase, an assumption which may not be entirely justified.

The behaviour of wheat proteinase towards ovalbumin finds again a parallel in the behaviour of papain [Willstätter & Grassmann, 1924], yeast proteinase [Grassmann & Dyckerhoff, 1928] and malted barley proteinase [Linderstrøm-Lang & Mill, 1929] towards this substrate; in no instance is the native protein hydrolysed. The albumin is however attacked by yeast proteinase which has been activated by cyanide while the denatured protein is degraded by papain without cyanide activation [Willstätter *et al.* 1926]. The above evidence of absence of hydrolysis of ovalbumin was secured either by alcohol or formaldehyde titration of the end products of hydrolysis. On the other hand, by measuring the disappearance of substrate, as indicated by the loss of coagulability of the ovalbumin, Hopkins & Kelly [1931] were able to demonstrate an optimum of *p*H 3·3–3·6 for the action of malted barley proteinase on the native protein. It is conceivable that the chemical changes accompanying the loss of coagulability are not of sufficient magnitude to influence a formaldehyde or alcohol titre. Northrop [1922], for example, obtained different results on measuring the effect of substrate concentration on the tryptic digestion of casein according to whether he measured the rate of disappearance of casein or that of amino-nitrogen liberation.

From the curves in Fig. 2 it is clear that wheat gluten is acted upon by the proteinase present in aqueous extracts of sprouted wheat, the reaction having an optimal range of *p*H 5·6–6·4 with a mean value of 6·0. This figure is slightly to the acid side of the isoelectric points of gliadin and glutenin as reported by Tague [1925], who found a value for gliadin of *p*H 6·5 and for glutenin of *p*H 6·8–7·0. The choice of gluten as a substrate is of course open to criticism: gluten is neither a pure protein nor simply a group of proteins. It contains in addition to gliadin and glutenin small quantities of fats and lipins, carbohydrate, mineral matter etc. A typical analysis of dried gluten is given below [Norton, 1906]:

	%
Fat or ether extract	4·20
Carbohydrate	9·44
Fibre	2·02
Mineral matter	2·48
Gliadin	39·09
Glutenin	35·07
Globulin (10% NaCl extract)	6·75
	99·05

Gluten was nevertheless employed as such, firstly on account of its unique position among proteins, secondly, because it is presumably the natural substrate for wheat proteinase, and thirdly, on account of its considerable technical importance. That the results shown in Fig. 2 are due to hydrolysis of the principal gluten proteins and not to the decomposition of concomitant substances such as fats and lipins is proved by the method of their determination: increases in titre (*b*) by formaldehyde titration can be due only to the formation of amino-acids or closely related compounds. Further, although there arises the possibility of the hydrolysis of other proteins such as wheat globulin or leucosin which remain in the mass of gluten, nevertheless the extent of this hydrolysis could have but little influence upon the final result on account of the extreme dilution of these proteins in the final mixtures. For example, assuming the amount of globulin in the sample of dry gluten used in the present experiments to have been 7 %, the concentration of the globulin in the final reaction mixtures could not at any time have exceeded 0·21 %. This corresponds to a final crude gluten percentage of 3. If then the curves in Fig. 3 are assumed to be the outcome of, say, wheat globulin hydrolysis, then not only are titre increases of considerable magnitude obtained at substrate concentrations of 0·21 % but appreciable increases still accrue at concentrations as low as or even lower than 0·007 % (corresponding to a gluten percentage of 0·1). Judging from the data obtained with edestin and gelatin such an interpretation is highly improbable.

Gluten hydrolysis by wheat proteinase does not appear to be affected by sodium chloride in concentrations up to 0·25 *M*, nor is the amount of substrate precipitate present in each mixture increased by the salt addition. Edestin, on the other hand, is precipitated by sodium chloride in acid solution and the rate of hydrolysis of edestin in the presence of this salt has been shown to depend upon the amount of protein remaining unprecipitated [Mounfield, 1936, 1]. The apparently obvious explanation that by precipitation the edestin is removed from the sphere of activity of the enzyme cannot, however, be accepted as the sole reason for the inhibitory effect, since precipitation of edestin can occur in certain mixtures containing acetate buffers only, particularly at about *p*H 5, without an equivalent reduction in rate of hydrolysis [Mounfield, 1936, 1, Fig. 1]. The edestin precipitated in sodium chloride solutions does not therefore appear to be identical with that formed by simple adjustment of an edestin solution to its isoelectric point. This suggests the formation of an edestin-sodium chloride complex which is not attacked by the proteinase. That the latter enzyme is capable of directly hydrolysing precipitated substrate is proved still further by the experiments on the relation between initial reaction velocity and gluten concentration. At all concentrations from 0·05 to 3·0 % the gluten appeared to be completely precipitated, yet with increase in gluten concentration there was continued increase in total titre (see Fig. 3).

Figs. 3 and 4 depict the results of parallel experiments on the variation in initial reaction velocity with substrate concentration using gluten and edestin, respectively. In the case of gluten the titre (*b*) curve reaches a limiting value at a substrate concentration of approximately 1·5 %, while for edestin the curve becomes almost horizontal at approximately 2 % substrate. The latter curve was published in an earlier communication [Mounfield, 1936, 1] but is here reproduced for convenience in reference. The substrate concentration at which half the limiting velocity is reached should be numerically equal to the Michaelis constant, K_m, a characteristic of the enzyme. For edestin this has already been reported as occurring at about 0·05 %. From Fig. 3 the value for gluten is seen to be approximately 0·25 % although in view of the complex nature of this

substrate and the probable interference of dipeptidase activity (see below) the precise meaning to be attached to this information is rendered even more obscure than in the case of edestin.

The curve in Fig. 3 showing increase in total titre ($a+b$) with variation in substrate concentration does not run parallel with that for titre (b), the ratio titre (a) : titre (b) increasing with increasing substrate concentration. In Fig. 4 however the curves for total titre and titre (b) approximate more closely in shape, the ratio total titre : titre (b) being almost constant at 2 : 1. In neither case is a limiting value reached within the limits of the experiment.

In this connexion it should be borne in mind that titre (b) represents principally the increase during hydrolysis in amino-acid-N together with a portion of the polypeptide amino-N while total titre includes also that portion of the polypeptide amino-N not measured in titre (b) and also any carboxyl groups liberated, for example, by ester scission [Richardson, 1934; 1935; Grünhut, 1919]. Ester scission is unlikely in the present instance and in any case would be very largely eliminated by the deduction of control values so that increases in titre (a) or titre (b) may safely be assumed to result from hydrolysis of protein only.

Now the mean pK' of most peptides is about 8·3 while that of amino-acids is about 9·6 [Richardson, 1934] so that peptide formation alone (if such should occur) would lead to increases in both titre (a) and titre (b). Amino-acid production on the other hand would result in increase in titre (b) without appreciable rise in titre (a). The distinction is of course by no means quantitative but merely indicates a tendency. The hydrolysis therefore of, say, glycylglycine ($pK'=7{\cdot}75$) to glycine ($pK'=9{\cdot}75$) or of leucylglycine ($pK'=7{\cdot}83$) to glycine and leucine ($pK'=9{\cdot}6$) should cause a decrease in titre (a), a comparatively large increase in titre (b) and a small increase in total titre. These changes were observed experimentally and are discussed below.

Thus the continued rise in total titre with increasing substrate concentration in Fig. 3 after titre (b) has attained an almost constant value suggests at first sight a continuance of polypeptide formation after amino-acid production has reached its maximum. Whether or not this be due to the activities of more than one enzyme it is difficult to state with certainty. In the gluten experiment with reaction conditions at pH 6 it is however probable that the proteinase and dipeptidase are active simultaneously, in which case a constant ratio between total titre and titre (b) cannot be expected. It is possible also for the dipeptidase to reach saturation point at a lower percentage of substrate than does the proteinase. In the case of edestin the reaction conditions (pH 4·1) would almost immediately cause the destruction of any dipeptidase.

It is therefore highly suggestive to discover that in this connexion there is no disturbance of the balance between polypeptide and amino-acid productions when edestin concentration is increased. There is an almost constant ratio between total titre and titre (b) (see Fig. 4), so that if produced the two curves should become horizontal at the same point. The activities of a single enzyme, namely, wheat proteinase, thus appear to account for the relevant facts.

The activation of wheat proteinase by cyanide using edestin as substrate has already been described [Mounfield, 1936, 2]. According to the hypothesis of Bersin [1933] and co-workers cyanide activates by reducing the S—S linkage of the inactive oxidized form of the enzyme to the SH form. This merely has the effect of increasing the quantity of active enzyme so that the course of any given proteolysis should not thereby be altered except in velocity. This reasoning is supported by the results of the experiments on the effect of cyanide on the relations between edestin concentration and initial reaction velocity (see Fig. 4).

The ratio total titre : titre (*b*) with cyanide is almost identical with the corresponding ratio without cyanide, namely, 2 : 1 in each case. This indicates quite clearly that cyanide caused no disturbance of the equilibrium between polypeptide and amino-acid production. The cyanide merely elevates both total titre and titre (*b*) curves to proportionate extents and does not induce a more complete hydrolysis of the split products.

The inability of cyanide to effect an increase in the rate of hydrolysis of gluten as measured by increase in total titre (see Fig. 3) is somewhat surprising since in most other respects this substrate behaves similarly to edestin. It is unusual to find that activation by cyanide depends upon the substrate. It is possible that the mechanism of attack by the enzyme is different in the case of gluten or it may be that the method of preparation of the substrate permits the introduction into the reaction mixture of reducing substances which completely activate the proteinase and render the cyanide ineffective.

Another explanation however presents itself. Since proteins most probably contain the S—S and SH type of linkages it is conceivable that they also are affected by cyanide in much the same manner as papain and related enzymes are considered to be affected. Such an assumption would explain not only the dependence of activation upon choice of substrate (gluten for example might be regarded as a fully "activated" protein, the addition of cyanide causing no further modification or increase in susceptibility) but it would also form the basis of a rational explanation of the extension of the range of specificity exhibited by cyanide-activated papain, cathepsin and yeast proteinase. According to Willstätter & Grassmann [1924] untreated papain attacks only gelatin, denatured albumin and possibly peptones whereas after activation native albumin, histones, protamines and even certain tripeptides are included within its range of substrates. An almost identical widening of substrate range occurs with cathepsin [Waldschmidt-Leitz *et al.* 1929] and yeast proteinase [Grassmann & Dyckerhoff, 1928]. Instead, therefore, of supposing that the enzyme is the activated component it is equally reasonable to consider the protein as being "activated" or rendered more susceptible to attack. Indeed, both enzyme and substrate may be affected in this way.

In spite of its inability to affect the increases in total titre in the gluten experiment cyanide does however cause a small but definite increase in titre (*b*) and a corresponding reduction in titre (*a*) (see Fig. 3). In accordance with the argument outlined above this disturbance in the equilibrium suggests that the quantity of amino-acid is augmented by a more complete hydrolysis of peptide linkages brought about in all probability by cyanide activation of the dipeptidase. That such is still possible to a small extent at *p*H 6 has already been demonstrated [Mounfield, 1936, 2]. Support is gained for this view from the data cited in Table I. In the absence of all substrate except that naturally present in the sprouted wheat extract by virtue of its method of preparation (probably wheat globulin and leucosin together with peptides etc.) slight activation by cyanide as measured by increase in total titre is again observed. Further, the same depression of titre (*a*) and increase in titre (*b*) is evident. The phenomenon is absent below about *p*H 5·5 but is prominent between *p*H 7 and 8 a range which covers the optimum for dipeptidase activity, to which the effect is no doubt due. The middle line in Fig. 3 is of course plotted from values obtained after deduction of such controls carried out in the presence of cyanide. Reference has already been made to the fact that precisely similar results were recorded during the measurement of the hydrolysis of glycylglycine and leucylglycine by wheat dipeptidase.

An unusual feature of this work has been the almost complete inability of the enzymes of sprouted wheat extract to attack either glutenin or gliadin as prepared by the methods outlined in the introduction to this paper. In the case of gliadin it is true there is evidence of a slight optimum at or near *p*H 5 but the extent of the hydrolysis in no way compares with that secured with other substrates. Doubling the concentration of substrate was without influence upon the titre increase, a fact which disposes of any error which might have arisen through the use of substrate concentrations below saturation point. The device of using a mixed substrate of glutenin and gliadin in equal proportions was also without avail—there appears to be no complementary relationship between the two proteins in this connexion.

It has been pointed out by Dakin & Dudley [1915] that contact with alkali, which causes racemization of proteins can render the protein molecule resistant to attack by proteolytic enzymes. This might conceivably be the explanation for the slight increase in the extent of hydrolysis of glutenin when prepared without contact with alkali, but it is certainly not responsible for the resistance shown by the gliadin used in these experiments.

Several possibilities suggest themselves but none appears to offer a satisfactory explanation of the phenomena. In the first place it is conceivable that by prolonged contact with reagents such as ethyl alcohol and acetic acid, necessary for their preparation, the proteins have been modified in such a way as to obstruct proteolysis. This however is contrary to commonly observed facts, since denaturation, coagulation and allied changes tend rather to render the protein structure more susceptible to attack. Secondly, the method of preparation may have introduced substances possessing power to inhibit proteolysis or, on the other hand, may have removed certain necessary activators. The latter two suggestions offer most promise, but so far work along these lines has proved unfruitful. Attempts to activate with cyanide, for example, have been unavailing.

Summary

1. Wheat proteinase hydrolyses gelatin optimally at *p*H 5·1 and wheat gluten at *p*H 6·0 but does not attack ovalbumin, and affects glutenin and gliadin only slightly.

2. Amino-acid production from gluten reaches a maximum at about 1·5% substrate concentration while half the maximum reaction velocity is reached at about 0·25% gluten. Sodium chloride is without effect upon gluten hydrolysis.

3. The effect on initial reaction velocity at *p*H 4·1 of variation in edestin concentration is explicable on the assumption that only wheat proteinase is active. With gluten as substrate and reaction conditions at *p*H 6 the end products appear to be capable of further modification by the participation of wheat dipeptidase in the reaction.

4. Activation of wheat proteinase by cyanide using edestin as substrate merely affects reaction velocity and does not bring about a more complete hydrolysis of the cleavage products.

5. No activation with cyanide is observed when gluten is employed as the substrate for the proteinase. The decrease in the ratio titre (*a*) : titre (*b*) may be attributed to a slight activation of the concomitant dipeptidase.

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CCXVIII. PROTEINASE SECRETION AND GROWTH OF *CLOSTRIDIUM HISTOLYTICUM*

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YOUNG cultures of *B. fluorescens liquefaciens* quantitatively secrete a proteinase into the culture medium, as was found by Virtanen & Tarnanen [1931; 1932] and Virtanen & Suolahti [1937]. In previous work on the proteinase of *Cl. histolyticum* Weil & Kocholaty [1937] reached a similar conclusion.

Recently Maschmann [1938, 1] reported on a bacterial proteinase (*B. botulinus*, *B. Welchii*) which he considers to be in the "intracellularly acting proteinase of anaerobic bacteria", differing from the proteinase which is secreted by the bacterial cell. In a later publication on *Cl. histolyticum*, the same author [Maschmann, 1938, 2] concludes that this micro-organism produces three distinct proteinases. These are somewhat vaguely differentiated according to their actions on different protein substrates and their behaviour towards cysteine and other activators. Two of these enzymes are believed to be "extracellular" in character, being secreted in the early stages of bacterial growth and to be indifferent towards, or even inhibited by, cysteine and other common activators. The third enzyme is considered to be "intercellular", and to differ from the others in its ability to hydrolyse clupein but not gelatin, and to be activated by —SH compounds. Maschmann claims that the activation phenomena observed by us on culture filtrates of *Cl. histolyticum* are due to this intracellular proteinase, which is freed only by autolysis of the bacterial cell.

Our previous work on this micro-organism has been extended and the results are still at variance with the conclusions reached by Maschmann. In cultures of *Cl. histolyticum*, both the bacterial growth and the proteolytic activity (initial and full[1]) of the cell-free bacterial filtrates, reach a maximum value within 24 hr. after inoculation. This is true regardless of the type of medium employed provided that the latter has *p*H near the neutral point. In all cases here investigated it was possible to activate the proteinase by means of Fe^{++} in combination with sulphydryl compounds. Thus, a proteinase activated by Fe^{++}-cysteine was found in filtrates from 6 hr. cultures, at a time when practically no bacterial autolysis had yet taken place. Similar activation was observed in filtrates prepared after incubation periods up to 12 days; Fe^{++}-cysteine likewise activated the intracellular proteinase obtained by destroying the bacteria themselves with a supersonic oscillator. On the basis of the results there appears no justification for assuming a difference between the intracellular proteinase and the proteinase secreted into the culture medium.

Cell-free filtrates obtained after incubation periods ranging from 12 to 48 hr. were found to hydrolyse both clupein and gelatin. In every case activation by

[1] By initial activity is meant the original proteinase activity without addition of activator; by full activity is meant the activity obtained in the presence of an Fe^{++}-SH activator. In the present work cysteine was used as the —SH source, underconditions described by Weil & Kocholaty [1937].

means of Fe^{++}-cysteine was observed. Maschmann states that the clupein-splitting enzyme is intracellular, and distinct from the gelatin-hydrolysing, extracellular enzymes. But its appearance in the culture filtrates (12 hr. incubation) before appreciable bacterial autolysis has occurred, indicates that it too is secreted and is probably identical with the gelatin-hydrolysing enzyme.

When cultures of *Cl. histolyticum* are incubated beyond the point at which maximum bacterial growth and proteolytic activity occur (24 hr.) a fairly rapid decrease in the activity of the proteinase takes place. This is not due to a temperature effect but appears to be related in some way to the autolytic processes. The stability of the bacterial proteinase depends also on the type of medium employed in the culture.

Experimental

The organism used was, as in previous work, a dissociated strain of *Cl. histolyticum* obtained from the American Type Culture Collection, No. 4872 [Hoogerheide, 1937]. The culturing was done as previously described, with the exception that the time of incubation was 24 hr. unless otherwise stated [Kocholaty & Hoogerheide, 1938]. The medium was in every case adjusted to *p*H 7·4 before inoculation. The estimation of proteolytic activity was carried out by measuring the liberation of free NH_2 groups from a gelatin substrate by the Van Slyke method. All results are expressed in terms of ml. 0·1 *N* base equiv. NH_2-groups liberated. Unless otherwise stated, the enzyme studies were made with cell-free solutions obtained by centrifuging and filtering the cultures through Seitz filters. Bacterial counts were made as previously described.

Secretion of proteinase

The relationship between bacterial growth and proteolytic activity is shown in Fig. 1. A horse meat broth medium (National Drug Co.) was inoculated with *Cl. histolyticum* and incubated at 37·5°. Samples were removed at intervals,

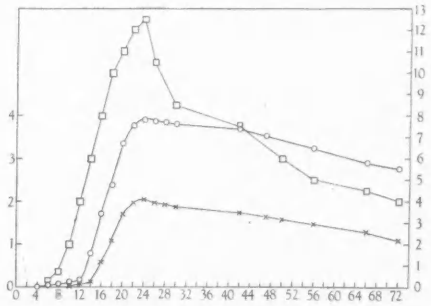

Fig. 1. Relation between growth and proteinase secretion of *Cl. histolyticum*. Abscissa: hr. bacterial growth after inoculation. Ordinate (left side): proteinase activity in ml. 0·1 *N* KOH. Ordinate (right side): no. of bacteria in 1 ml. $\times 10^{-5}$. □ = no. of bacteria. ○ = full activity of *Cl. histolyticum* proteinase. × = initial activity of *Cl. histolyticum* proteinase.

filtered through Seitz filters and both initial and full activities measured. Bacterial counts were made at the same time. Both initial and full activities of the proteinase in the culture filtrates increased with increase in bacterial count up to a maximum at about 24 hr. After this a sharp drop occurred in the number of bacteria present and the initial and full proteolytic activities also decreased, but

at a slower rate. These results indicated the improbability of the proteinase being liberated into the medium as a result of bacterial autolysis. If such were the case an increase in the proteinase activity would be expected to occur after the 24 hr. maximum. Such an increase has never been observed. The proteinase which is activated by Fe^{++}-SH is already present in the filtrates 6 hr. after the inoculation, and increases rapidly with increase in bacterial count. This is doubtless an example of true bacterial enzyme secretion. In contrast to this finding, Maschmann claims that just this particular proteinase which can be activated by Fe^{++}-SH is the "intracellular proteinase, which is liberated only by autolytic processes occurring after the death of the bacteria, and which makes its appearance in the culture medium at a relatively late stage of incubation".

When the bacteria are separated by centrifuging and Seitz filtration from a culture which has not yet reached its maximal growth (15 hr.), it is found that the cell-free filtrate contains practically the whole of the proteolytic activity. The bacteria themselves attack gelatin only slightly.

Example: a 100 ml. portion of 3% neopeptone, *p*H 7·4, was inoculated with *Cl. histolyticum*, and after 15 hr. incubation at 37·5° was passed through a Seitz filter. One ml. of the bacteria-free filtrate gave, in 20 hr. incubation with gelatin under the usual conditions, an initial activity of 1·56 and a full activity of 2·62 (ml. 0·1 *N* alkali). The entire mass of bacteria removed by centrifuging and filtration was washed, suspended in saline and subjected to the action of a supersonic oscillator until cell destruction was about 80% complete. The proteolytic activity of the whole mass of broken-up bacteria, measured against gelatin as above was: initial, 0·26; full, 0·68. The proteolytic activity of the bacterial mass was only about 0·3% of that of the whole filtrate. That the bacteria themselves do show a slight proteolytic activity may be due to the fact that they still contain small amounts of preformed, unsecreted proteinase. Since in a 15 hr. culture rapid cell proliferation is still taking place and little autolysis has occurred, the fact that almost the entire amount of proteinase is found in the filtrate is further evidence that a true secretion of enzyme as expressed both by initial and full activities has occurred. The bacteria themselves contain a polypeptidase and a dipeptidase which pass into the culture medium only on autolysis and which are not secreted by young cells under conditions in which the proteinase is secreted. This fact also supports the idea that the latter is liberated not by autolysis but by a truly secretory process.

In a second, similar experiment, 4 l. 2% neopeptone were inoculated with *Cl. histolyticum* and incubated at 37·5° for 20 hr. The bacteria were collected by centrifuging, washed thoroughly with physiological saline and finally suspended in 10 ml. saline. 1 ml. of this suspension gave an initial activity against gelatin of 0·98 ml., and a full activity of 1·21 ml. When the bacterial suspension was first treated with the supersonic oscillator until 80% cell destruction occurred, 0·2 ml. gave the following activities: initial 1·31 ml.; full, 3·29 ml. Also in this experiment the activation behaviour of the proteinase obtained from the bacteria themselves did not differ from that previously observed for the enzyme secreted into the culture medium.

If the changes occurring in the bacterial count and the proteolytic activity (both initial and full) in a *Cl. histolyticum* culture are determined from time to time one finds, as previously shown, that a maximum occurs at about 24 hr., after which a steady decrease takes place. If to such an incubation mixture in which the bacterial count has passed its maximum and is decreasing rapidly, one again adds fresh sterile culture medium, the bacterial count rises and reaches a new maximum at 24 hr. Simultaneously both the initial and full proteolytic

activities of the medium also increase to a new maximum. Further incubation beyond this point results again in a decrease in both bacterial count and proteolytic activity. A typical experiment is shown in Fig. 2. Enzyme activities were determined as before, on cell-free Seitz filtrates of the culture medium.

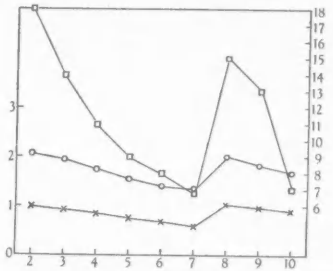

Fig. 2. Relation between bacterial growth and proteinase secretion. Abscissa: days of incubation. Ordinate (left side): proteinase activity in ml. 0·1 *N* KOH. Ordinate (right side): number of bacteria in 1 ml. $\times 10^{-5}$. □ = no. of bacteria. ○ = full activity of *Cl. histolyticum* proteinase. × = initial activity of *Cl. histolyticum* proteinase. Addition of new culture medium on the 7th day of bacterial growth.

Hydrolysis of clupein by the proteinase, and its activation by Fe^{++}-SH

When a bacteria-free culture filtrate of *Cl. histolyticum* (12 hr. growth) was allowed to act on gelatin or clupein, both substrates were split by the proteinase. In each case activation of the proteinase was obtained by addition of Fe^{++}-SH. Since in 12 hr. maximal bacterial growth has not nearly been reached (see Fig. 1) although clupein and gelatin are decomposed by the bacterial filtrate, this effect must be due to a proteinase which is secreted. After maximal growth has passed and autolysis of the bacteria occurs there is no increase in enzyme activity, either initial or full, towards clupein, as reported by Maschmann, but rather a decrease (Table I).

Table I. *Hydrolysis of clupein by* Cl. histolyticum *proteinase*

Cl. histolyticum grown in a solution of 3% heart infusion broth (Difco-standardized) at *p*H 7·4. After 12, 24 and 48 hr. growth at 37·5° samples were taken out and the Seitz-filtered enzyme solutions used for the exp. Determination: 2 ml. enzyme solution, 1 ml. cysteine-HCl (10 mg., neutralized), 0·6 ml. *M*/10 $FeSO_4$, or 1·6 ml. water, 2 ml. citrate-phosphate buffer *p*H 7·0 (McIlvaine) and 2 ml. 6·6% gelatin (neutralized) or 2 ml. of clupein sulphate (= 50 mg. adjusted to *p*H 7·0). Digestion time was 5 hr. at 37·5°.

Age of bacterial culture (hr.)	Activity of the proteinase			
	Gelatin substrate		Clupein substrate	
	Initial	Full	Initial	Full
12	0·28	0·51	0·26	0·39
24	0·57	1·14	0·38	0·73
48	0·40	0·85	0·27	0·55

Since the activation behaviour, as well as the secretion, was found to be practically identical with respect to clupein and gelatin hydrolysis, there seems to be no reason to assume that the clupein-splitting enzyme differs from the one attacking gelatin, as Maschmann claims.

Stability of the proteinase

The decrease in both the initial and the full proteolytic activities after maximal bacterial growth has occurred is surprising. This decrease has been studied in three different culture media. It was found that the highest stability is shown in a casein medium. In neopeptone the stability is less, and about equal to that in horse meat broth (see Figs. 3 and 4). It seemed likely that the decrease

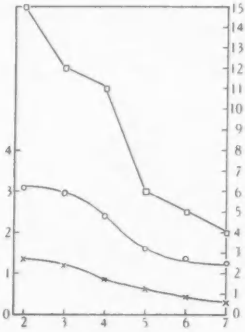

Fig. 3.

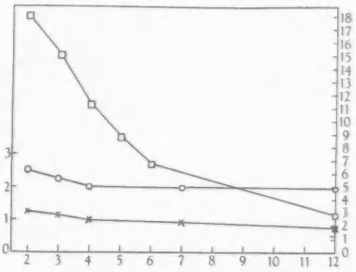

Fig. 4.

Fig. 3. Stability of *Cl. histolyticum* proteinase on neopeptone as culture medium. Abscissa: days of incubation. Ordinate (left side): proteinase activity in ml. 0·1 *N* KOH. Ordinate (right side): number of bacteria in 1 ml. $\times 10^{-5}$. □ = no. of bacteria. ○ = full activity of *Cl. histolyticum* proteinase. × = initial activity of *Cl. histolyticum* proteinase.

Fig. 4. Stability of *Cl. histolyticum* proteinase on casein as culture medium. Abscissa: days of incubation. Ordinate (left side): proteinase activity in ml. 0·1 *N* KOH. Ordinate (right side): no. of bacteria in 1 ml. $\times 10^{-5}$. □ = no. of bacteria. ○ = full activity of *Cl. histolyticum* proteinase. × = initial activity of *Cl. histolyticum* proteinase.

in the activity of the secreted proteinase in the culture medium might be due to inactivation during the long periods of incubation at 37·5°. Table II shows that this is not the case, since cell-free filtrates kept at 37·5° retain their proteolytic activity much longer than do unfiltered portions of the same incubation mixture. It is probable that the decrease is related to some process occurring during the autolysis of the bacteria.

Table II. *Stability of* Cl. histolyticum *proteinase*

Horse meat broth medium, 24 hr. culture; one portion (A) of the culture was allowed to stand at 37·5° without previous removal of the bacteria; a second portion (B) was freed from bacteria by Seitz filtration and also incubated at 37·5°; a third portion (C) was freed from bacteria and allowed to stand in the ice box. Enzyme determinations were made every 24 hr.

Days of incubation at 37·5°	Proteinase activity					
	A		B		C	
	Initial	Full	Initial	Full	Initial	Full
0	1·24	2·14	1·24	2·14	1·24	2·14
1	0·58	1·42	1·20	2·88	1·35	2·40
2	0·40	1·17	1·20	2·22	1·32	2·38
3	0·32	0·97	1·20	2·18	1·36	2·41
4	0·22	0·76	0·97	2·12	1·37	2·42

SUMMARY

The proteinase of *Cl. histolyticum* is liberated into the culture medium by secretion from the living bacteria, and is activated by Fe^{++}-cysteine under all conditions. On the basis of its activation behaviour, and its effect on various protein substrates, it appears to be identical with the proteinase obtained by breakdown of the bacterial cell.

The authors wish to express their gratitude to Dr Ellice McDonald, Director, for his interest and support throughout the work.

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CCXIX. STUDIES ON THE ENDO-ENZYMES, PARTICULARLY THE PEPTIDASES, OF *CLOSTRIDIUM HISTOLYTICUM*

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The first noteworthy work on bacterial peptidases as endo-enzymes was that of Virtanen & Tarnanen [1931; 1932, 1, 2] on *B. fluorescens liquefaciens*. Later, Gorini *et al.* [1932] and Gorbach & Pirch [1936–7] reported on the same subject. These various authors agree that the bacterial peptidases are present exclusively within the bacterial cell, are not secreted and show optimal activity at an alkaline reaction. Also in no instance has it been possible to demonstrate the presence of a carboxypeptidase in the bacterial cell.

In all of the above-mentioned work use was made of toluene autolysis to liberate the peptidases from the intact cell. Investigations carried out in this laboratory on the strict anaerobe, *Cl. histolyticum*, have shown that this time-consuming method of autolysis can be dispensed with to advantage by use of a supersonic oscillator to disrupt the bacterial cells. With this apparatus it is possible to break up the bacterial cells in a very short time, which is of particular advantage in view of the extreme instability of the bacterial peptidases in solution. When the tissue debris is removed from such preparation by filtration, a cell-free, water-clear fluid containing considerable quantities of peptidases is obtained. In this way it was shown that young cultures of *Cl. histolyticum* contain a polypeptidase with *p*H optimum at about 8·7, and a dipeptidase with an optimum at *p*H 7·6. All attempts to detect a carboxypeptidase failed, and it appears fairly certain that such an enzyme does not exist in bacteria. The bacterial polypeptidase is activated to a considerable extent by Mg^{++}, a phenomenon previously described by Johnson *et al.* [1936] for the animal polypeptidase, leucylpeptidase. The bacterial dipeptidase appears to be indifferent toward Mg^{++}.

Among the deamidases, we were especially interested in the enzyme which deaminizes *l*-aspartic acid. The deamination of this amino-acid by suspensions of *Cl. histolyticum* occurs optimally at *p*H 7·6 with formation of succinic acid as previously shown by Cook & Woolf [1928]. The presence of an aspartase could not be demonstrated, although previously this enzyme had been found in suspensions of *B. coli commune* by Quastel & Woolf [1926], and in cell-free preparations of *B. fluorescens liquefaciens* by Virtanen & Tarnanen [1932, 1, 2]. We were however able to show the presence of other deamidases, which in a weakly alkaline medium attack glycine, *l*-leucine, *d*-alanine, *d*-glutamic acid and *l*-tryptophan with liberation of NH_3.

In a previous publication Weil & Kocholaty [1937] suggested that the peptidases of *Cl. histolyticum* might be secreted by the intact cells. This idea was advanced because of the fact that no polypeptidase could be found in bacteria which had been autolysed with toluene. However, at that time determinations were made on very small quantities of bacteria and the activating effect of Mg^{++}

was unknown, so that the peptidase in question may have been present in the inactive form. The results now reported definitely indicate that these enzymes are not secreted, but must be classed as endo-enzymes.

Experimental

The micro-organism used, as in previous work, was the dissociated strain of *Cl. histolyticum* described by Hoogerheide [1937]. The culture medium used in all cases was a 2% solution of neopeptone (Difco standardized), adjusted before inoculation to *p*H 7·4. After an incubation period of 18–20 hr. at 40°, the bacteria were centrifuged down, washed carefully, resuspended in saline and used at once. Such a suspension contains a negligibly small amount of spores and cellular debris, most of the bacteria being intact. The *p*H of the suspensions was always 7·0. When studies were to be made on cell-free material the washed bacteria were disrupted in the supersonic oscillator, after which the cellular debris was removed by Seitz filtration. The enzyme solutions obtained in this way were extremely unstable.

Under the conditions employed, a 10 min. treatment of a bacterial suspension in the supersonic oscillator resulted in a destruction of about 50% of the cells. This could be increased to 80–85% by a further treatment of 10 min. In order to avoid destruction of the enzymes the disruption was never carried beyond that point. This method for preparing bacterial enzyme solutions is advantageous not only because the cellular disruption is accomplished rapidly but also because it avoids the use of toluene which is sometimes harmful to enzymes. However, because of the formation of slimy material, bacterial suspensions treated in this way are very difficult to filter.

The estimation of peptidase activity was carried out by measuring the increase in COOH or NH_2 groups produced in the substrates leucylglycine and leucylglycylglycine, using respectively the method of Foreman [1920], or of Van Slyke. The estimation of NH_3 was by the usual distillation method.

p*H optimum of the peptidases*

To 1 ml. portions of the above described cell-free extracts were added 3 ml. citrate-phosphate buffer (McIlvaine—for the higher *p*H values KOH was also added); 5 ml. *M*/5 leucylglycine or 5 ml. *M*/5 leucylglycylglycine, adjusted to the proper *p*H, were added as substrates. The mixtures were incubated for 7 hr. at 40°. *p*H estimations made before and after the incubation period showed practically no change. The increase in free NH_2 groups was measured by the Van Slyke method, the results being expressed in ml. of 0·1 *N* base (Figs. 1 and 2). It should be pointed out that in all experiments reported the polypeptidase was accompanied by dipeptidase, since no attempt was made to separate the two enzymes. The values for polypeptidase activity are therefore only approximations.

Activation of the polypeptidase by Mg^{++}

Reaction mixtures were prepared consisting of 1 ml. cell-free enzyme solution prepared as described above, 5 ml. *M*/5 leucylglycine or leucylglycylglycine previously adjusted to the optimal *p*H, 3 ml. of citrate-phosphate buffer at the optimal *p*H and 1 ml. of water or $MgCl_2$ solution, the last to make the final molarity in the reaction mixtures 0·005–0·020. The mixtures were incubated at 40° for 7 hr., the COOH increase being measured by the method of Foreman. Results (Fig. 3) are expressed in ml. of 0·1 *N* KOH.

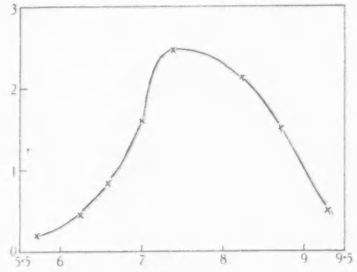

Fig. 1.

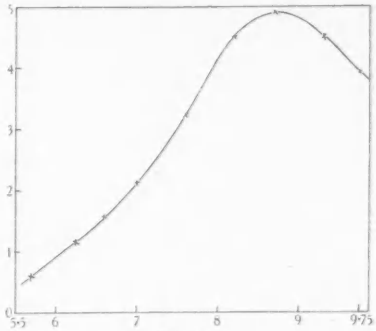

Fig. 2.

Fig. 1. *p*H optimum of *Cl. histolyticum* dipeptidase. Abscissa: *p*H. Ordinate: dipeptidase activity in ml. 0·1 *N* KOH.

Fig. 2. *p*H optimum of *Cl. histolyticum* polypeptidase. Abscissa: *p*H. Ordinate: polypeptidase activity in ml. 0·1 *N* KOH.

Fig. 3 shows the effect of conc. of Mg^{++} on the activity of *Cl. histolyticum* polypeptidase. Table I shows some results obtained with the bacterial dipeptidase at *p*H 7·6, and with the polypeptidase at *p*H 8·7. The Mg concentration was 0·01 *M* in the final reaction mixture. Comparative determinations were made using both a suspension of intact cells, and a cell-free enzyme solution prepared by the supersonic method.

Table I. *Effect of Mg^{++} on the activity of* Cl. histolyticum *peptidases*

	Dipeptidase activity hr. incubation				Polypeptidase activity hr. incubation			
Enzyme source	1	4	7	24	1	4	7	24
Intact cells	—	—	—	0·91	—	—	—	1·24
Intact cells + Mg^{++}	—	—	—	0·90	—	—	—	2·00
Disrupted cells	0·55	1·18	2·23	—	1·24	2·54	3·41	—
Disrupted cells + Mg^{++}	—	1·15	—	—	2·04	—	3·76	—

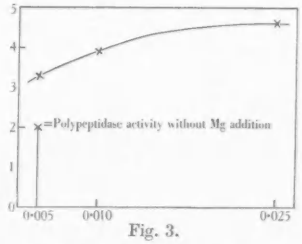

Fig. 3.

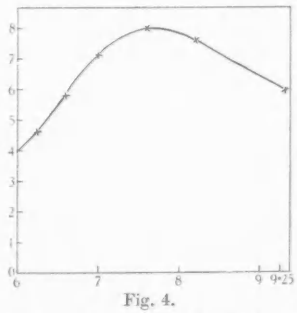

Fig. 4.

Fig. 3. Activation of *Cl. histolyticum* polypeptidase by Mg^{++}. Abscissa: molarity of $MgCl_2$ added. Ordinate: polypeptidase activity in ml. 0·1 *N* KOH. ×: polypeptidase activity without Mg addition.

Fig. 4. *p*H optimum of *l*-aspartic acid deaminase (*Cl. histolyticum*). Abscissa: *p*H. Ordinate: ml. *N*/70 NH_3 liberated.

It is apparent that the bacterial dipeptidase is indifferent toward the presence of Mg^{++}. The extensive activation of the polypeptidase by Mg^{++} suggests that this enzyme might be largely inactivated by dialysis. Addition of Mg^{++} to such dialysed preparations should reactivate the enzyme, as was shown by Johnson *et al.* [1936] in the case of the intestinal leucylpeptidase. However, the polypeptidase preparations obtained were too unstable to test this point.

Cell-free Seitz filtrates, obtained from young actively-growing cultures (15 hr.) of *Cl. histolyticum* in which little autolysis has occurred, contain practically no dipeptidase or polypeptidase. Also from Table I it is seen that young washed intact suspensions of the bacteria show little peptidase action unless the incubations are carried out for long periods of time (24 hr.). The activity observed under such conditions is probably the result of enzyme liberation by autolysis, since toluene was present as a preservative during the long incubation period. But if the bacteria are previously broken up by the supersonic oscillator, high peptidase activity is observed even in short incubations. Such results indicate that these enzymes are endocellular, and are liberated only after destruction of the cell membrane by autolysis or other means.

Tests made under the same conditions for the presence of a bacterial carboxypeptidase, using chloroacetyl-*l*-tyrosine as substrate at *p*H 7·4 and 8·4, and with and without added Mg^{++}, were negative in each case.

Deamination of l-*aspartic acid*

For establishing the *p*H optimum of this enzyme, reaction mixtures were prepared consisting of 2 ml. bacterial suspensions (treated with the supersonic oscillator but not filtered), 2 ml. *M*/10 *l*-aspartic acid and 5 ml. citrate-phosphate buffer. The incubation period was 20 hr. at 40°. The results are shown in Fig. 4 in terms of ml. *N*/70 NH_3 formed. The *p*H optimum for the deamination was near 7·6.

Similar reaction mixtures, extending over the same *p*H range, but containing 2 ml. *M*/10 fumaric acid and 10·7 mg. NH_4Cl in place of the *l*-aspartic acid (total volume, 11 ml.) gave no evidence of aspartic acid synthesis after 20 hr. incubation at 40°. No appreciable decrease in free NH_3 occurred, indicating that the enzyme aspartase was absent.

In another experiment a fairly strong disrupted bacterial suspension was allowed to stand at *p*H 7·6 for 36 hr. with 4 ml. *M*/10 aspartic acid (40°). After filtration, 10% H_3PO_4 was added, and the solution extracted several times with ether. The ether extract was evaporated to dryness, taken up in water, and treated with animal charcoal. On evaporation a crystalline material, M.P. 185°, was obtained. A mixed M.P. with pure succinic acid showed no depression. For further identification, the Pb salt was prepared, using Pb acetate. This salt was obtained in its characteristic form, small whetstone-shaped crystals arranged in rosettes. The deamination of aspartic acid yields succinic acid, as previously reported by Cook & Woolf [1928].

Deamination of other amino-acids

Because of the scarcity of enzyme material, it was not possible to determine the *p*H optima of the deamidases which attack other amino-acids. However, it could be shown that enzyme solutions obtained by the disruption method from *Cl. histolyticum* do bring about aerobic deamination of glycine, *l*-leucine, *d*-alanine, *d*-glutamic acid and *l*-tryptophan at *p*H 7·8. To 3 ml. enzyme solution

were added 2 ml. $M/10$ amino-acid solution and 5 ml. citrate phosphate buffer (McIlvaine). After 48 hr. incubation at 40°, the NH_3 liberated was determined by the distillation method. The results are shown in Table II.

Table II. *Deamination of amino-acids by* Cl. histolyticum *enzymes*

Amino acid	ml. $N/70$ NH_3 formed after 48 hr.
Glycine	4·34
d-Alanine	3·68
l-Leucine	4·86
d-Glutamic acid	5·70
l-Tryptophan	2·14

Intracellular nature of the enzymes

All attempts to show the presence of the above enzymes (dipeptidase, aminopolypeptidase, deaminases) in cell-free filtrates obtained from young rapidly growing cultures were unsuccessful. In agreement with other authors it must be concluded that these enzymes are not secreted during bacterial growth, but are liberated only upon autolysis of the cells.

Summary

1. Solutions obtained by disrupting *Cl. histolyticum* suspensions with a supersonic oscillator contain a dipeptidase with a *p*H optimum of 7·6, and a polypeptidase acting optimally at *p*H 8·7.

2. The polypeptidase is activated by Mg^{++}, while the dipeptidase is unaffected.

3. The preparations contain also enzymes which attack various amino-acids with liberation of NH_3.

4. All of the above enzymes are endo-enzymes, and are not secreted by the intact bacteria.

The authors wish to express their thanks to Dr Ellice McDonald, Director, for his interest and support throughout this work.

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CCXX. ENZYMIC ADAPTATION IN *CLOSTRIDIUM HISTOLYTICUM*

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Recently Kocholaty & Hoogerheide [1938] described an example of the adaptation of certain of the endo-enzymes of the strict anaerobe *Cl. sporogenes*: by varying the *p*H of the culture medium a considerable shift in the *p*H optimum of some of the dehydrogenases could be produced. It has now been found that under specified conditions the *p*H optimum of the proteinase secreted by this micro-organism can be shifted, and that, by repeated subculturings on gelatin or casein, specific proteinases are developed which will hydrolyse gelatin, but not casein, and *vice versa*.

Experimental

The methods used in preparing the *Cl. histolyticum* proteinase and estimating its activity were the same as previously described [Kocholaty *et al.* 1938]. Casein (Hammarsten) and gelatin (Coignet Gold Label) were used as culture media, and as substrates for proteinase activity determinations. Bacteria-free Seitz filtrates of the incubated cultures were used in the enzyme studies.

Shift in the p*H optimum of the proteinase*

The proteinase secreted by *Cl. histolyticum* grown at *p*H 7·2–7·4 on the common media, such as neopeptone, horse meat broth, gelatin or casein, has a *p*H optimum of 7·0 as found by Weil & Kocholaty [1937]. The same holds for the proteinase secreted by various other types of bacteria. When *Cl. histolyticum* is grown on casein medium at *p*H 6·0 for 20 hr. the secreted proteinase is no longer optimally active at *p*H 7·0 but at about *p*H 6·7. During the 20 hr. incubation, the *p*H of the culture medium increases from 6·0 to 6·7 or 6·8. If the culture medium

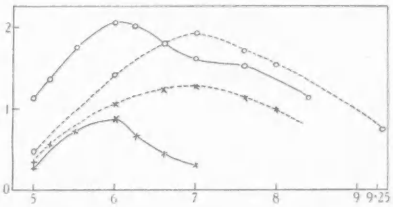

Fig. 1. *p*H optimum of *Cl. histolyticum* proteinase. Abscissa: *p*H. Ordinate: proteinase activity in ml. of 0·1 *N* KOH. ——=in acid medium (3% casein; 1% glucose). -----=in neutral medium (3% casein). ○=full activity. ×=initial activity. Age of the culture: 20 hr.

is held at a definitely acid reaction during the whole 20 hr. of incubation a still larger shift in the *p*H optimum of the secreted proteinase takes place. A very simple method for achieving this effect is to add glucose to the culture medium. When *Cl. histolyticum* is grown on casein (3%) in the presence of glucose (1%),

extensive gas formation occurs as a result of sugar breakdown, and the *p*H of the culture medium drops, because of the formation of acids, from *p*H 7·4 to about 5·8 toward the end of the incubation period. The *p*H optimum of the proteinase found in filtrates from such cultures is now about 6·0. These facts are illustrated in Fig. 1. It will be seen that identical shifts occur in the *p*H optima both of the initial and the full (with cysteine plus Fe^{++}) proteinase activities.

This simple example of enzymic adaptability can be made still more striking. If a solution of 3% casein at *p*H 7·4 is inoculated with a culture of *Cl. histolyticum* grown on casein plus glucose as described above, the proteinase secreted after 20 hr. growth is again found to be optimally active at *p*H 7·0 (both initial and full activities). It is evident that the nature of the proteinase secreted by the bacteria adjusts itself very easily and rapidly to changes in the culture medium.

The production of specific proteinases by Cl. histolyticum

The proteinase of *Cl. histolyticum* readily attacks casein, gelatin, horse meat broth, egg albumin, neopeptone and clupein among the media investigated. The hydrolysis, in every case, is activated by SH-heavy metal complexes, for example, by cysteine plus Fe^{++}. When the bacteria are grown on casein or gelatin the secreted proteinase attacks both casein and gelatin to about the same extent (see Table I, Exp. 1).

Among the easily available proteins of high mol. wt., casein and gelatin show the greatest divergence in their amino-acid compositions. For this reason these two proteins were chosen for the following experiments: the greater the difference in the proteins studied, the easier it should be to establish a specific enzyme adaptation in the proteinase secreted by *Cl. histolyticum* when repeatedly subcultured on the respective proteins. The main differences in the amino-acid compositions of casein and gelatin are shown below, the values being those given by Hawk & Bergeim [1931].

Amino-acid	% in gelatin	% in casein
Glycine	25·5	0·4
l-Hydroxyproline	14·1	0·2
d-Valine	0·0	7·9
d-Hydroxyglutamic acid	0·0	10·5
l-Tyrosine	0·01	6·5
l-Tryptophan	0·0	2·2

By repeated transfers of *Cl. histolyticum* cultures from gelatin to gelatin, and from casein to casein medium, an attempt was made to develop specificity in the secreted proteinase for one or the other of these proteins. The transplantations were made as follows: inoculations of *Cl. histolyticum* from the stock culture were made in test tubes containing 10 ml. 10% gelatin or 10% casein. After bacterial growth had become well established,[1] 1 ml. portions of these incubation mixtures were transferred into fresh 10% gelatin and casein media, respectively. This operation was repeated 6 times, always transferring from gelatin to gelatin, and from casein to casein. The incubation period during these

[1] It was frequently found that *Cl. histolyticum* did not grow readily on gelatin medium. When ordinary gelatin was used an entire week often elapsed before bacterial growth began. This induction period could however be shortened by using a gelatin medium which had first been partially digested by trypsin, and then sterilized. Usually after one subculture on such trypsin-digested gelatin, the bacteria became sufficiently adapted so that satisfactory growth and enzyme secretion could be achieved on ordinary (not previously decomposed) gelatin. With casein, on the other hand, good growth and enzyme secretion were usually obtained after the first inoculation, no extensive induction period occurring.

sub-culturings varied from 24 to 48 hr., satisfactory bacterial growth occurring within this time. After the 6th transfer, larger amounts of 3% gelatin and 3% casein were inoculated with the respective cultures, and incubated for 24 hr. The solutions obtained after removal of the bacteria by Seitz-filtration were used in the enzyme studies.

Table I. *Formation of specific proteinases by* Cl. histolyticum

	Enzyme ...	G		G		C		C	
	Substrate ...	Gelatin		Casein		Gelatin		Casein	
1	Initial activity	1·47		1·36		1·20		1·12	
	Full activity	2·51		2·69		2·34		2·43	
			*		*		**		**
2	Initial activity	1·18	1·12	0·38	0·54	0·46	0·70	1·00	0·97
	Full activity	2·16	2·00	1·88	1·81	1·80	1·79	2·27	2·24
3	Initial activity	1·28	1·20	0·00	0·40	0·00	0·37	1·18	1·12
	Full activity	2·50	2·68	1·20	1·28	1·47	2·00	2·32	2·41
4	Initial activity	0·30		0·18		0·24		0·41	
	Full activity	0·59		0·36		0·46		0·89	

Explanation of Table I:

G = Enzyme solution obtained by growing *Cl. histolyticum* on gelatin medium.
C = Enzyme solution obtained by growing *Cl. histolyticum* on casein medium.

Activity determinations were made on cell-free bacterial filtrates using gelatin and casein as substrates. Reaction mixtures contained 2 ml. filtrate, 3 ml. 6·6% casein or gelatin adjusted to *p*H 7·0, 5 ml. citrate-phosphate buffer (McIlvaine) at *p*H 7·0 and 1·6 ml. water (for initial activity) or 1 ml. neutralized cysteine-HCl (10 mg.) plus 0·6 ml. *N*/10 $FeSO_4$ (for full activity). Incubation time was 20 hr. at 40°. Estimations were made by the Van Slyke method, results being expressed in ml. 0·1 *N* base equiv. to the $-NH_2$ groups liberated.

Exp. 1. Comparison of proteinases obtained in casein and gelatin media after one inoculation. The bacterial filtrate was prepared from a culture of *Cl. histolyticum* grown for 24 hr. at *p*H 7·4 on 3% gelatin or casein.

Exp. 2. Specificity of proteinases obtained after 6 transplantations on 10% casein and 10% gelatin.

Column * = addition of 15 mg. *d*-valine, 13 mg. *l*-tyrosine, 4·3 mg. *l*-tryptophan and 21 mg. *d*-hydroxyglutamic acid to 10 ml. enzyme solution before activity determination.

Column ** = Addition of 50 mg. glycine and 28 mg. *l*-hydroxyproline to 10 ml. of enzyme solution before activity determination.

Exp. 3. Same as Exp. 2 except that 12 successive transplantations were made.

Exp. 4. Loss of specificity of *Cl. histolyticum* proteinases. Casein-trained bacteria (12 transplantations) were grown for 24 hr. on 100 ml. 3% gelatin supplemented with 150 mg. *d*-valine, 130 mg. *l*-tyrosine, 43 mg. *l*-tryptophan, and 210 mg. *d*-hydroxyglutamic acid. Gelatin-trained bacteria (12 transplantations) were grown for 24 hr. on 100 ml. 3% casein supplemented with 500 mg. glycine and 280 mg. *l*-hydroxyproline. After Seitz filtration, the enzyme solutions thus obtained (designated C and G, respectively) were tested for activity as described above.

As shown in Table I, Exp. 2, small differences are evident, after 6 transplantations, in the specificities toward gelatin and casein exhibited by enzymes developed respectively in gelatin and casein media. Particularly with reference to the initial activity, the gelatin-trained enzyme has lost some of its ability to hydrolyse casein, while the casein-trained enzyme attacks gelatin less readily than it does casein. By adding to the gelatin and casein enzymes, respectively, the more important amino-acids missing in casein and gelatin a slight increase in the initial proteinase activity towards these substrates is observed.

The transplantation of the above gelatin and casein cultures was continued as before, from gelatin to gelatin, and from casein to casein, until a total of 12 sub-culturings had been made. Then larger amounts of 3% gelatin and 3% casein media were inoculated, incubated for 24 hr., and the bacteria removed by Seitz filtration. Enzyme studies on the cell-free filtrates gave the results shown in Table I, Exp. 3. In the absence of added activator, the gelatin-trained enzyme

is no longer able to hydrolyse casein, and the casein-trained enzyme does not attack gelatin. Rather remarkable is the fact that addition of the activator, cysteine-Fe^{++}, reduces this specificity, so that the gelatin-enzyme now hydrolyses casein, and vice versa, the casein-enzyme attacks gelatin. Interesting also is the confirmation of the observation reported in Table I, Exp. 2, that the gelatin-enzyme can be somewhat activated towards casein by addition of the amino-acids missing from the latter, but present in gelatin. The same holds, *mutatis mutandis*, for the casein-trained enzyme.

When casein is inoculated with *Cl. histolyticum* which has been carried through 12 sub-culturings on gelatin, no bacterial growth takes place even in 2 weeks of careful incubation. The same holds true when gelatin is inoculated with the casein-trained bacteria. However, when the culture medium consists of gelatin plus those amino-acids which are missing from this protein but present in gelatin, the casein-trained bacteria will develop a fair growth in several days. Likewise the gelatin-trained bacteria will slowly grow on a casein medium, supplemented by the amino-acids absent from the latter but present in gelatin. As shown in Table I, Exp. 4, the enzymes secreted in these latter cases are able to attack both casein and gelatin again, although not very strongly.

Discussion

For the sake of brevity, the specific enzyme obtained by repeated sub-culturings on gelatin is termed "gelatinase", and that obtained in casein medium, "caseinase".

The results just described are of considerable interest from several points of view. By systematically training *Cl. histolyticum* on media such as casein or gelatin it is possible to develop two distinct proteinases of such extraordinary specificity that they can sharply differentiate between casein and gelatin. Also by simply culturing the casein-trained bacteria on a gelatin medium supplemented by those amino-acids missing from gelatin but present in casein, an enzyme is produced which again attacks both proteins. The same result is achieved by growing the gelatin-trained bacteria on casein supplemented by amino-acids missing from this protein but present in gelatin. Such results indicate that the specificity of these proteinases must be of very labile quality, depending only on the nature of the proteins used in their production. Addition of simple amino-acids to the culture medium, in fact, suffices to cause the specificity differences to disappear. The results presented in Table I, Exp. 4, show how easily these "trained" bacteria, and their secreted proteinases, can revert to their original state.

Of particular interest is the behaviour of the specific proteinases toward cysteine plus Fe^{++}. In the absence of this activator, the "gelatinase" and the "caseinase" fail to attack casein and gelatin, respectively, but both substrates are hydrolysed by each of these enzymes when the activator is present. There are two possible explanations for this SH-Fe^{++} effect.

1. The initial activity of the proteinase undergoes an activation in the sense that an extension of the field of specificity of the enzyme occurs. In other words, SH-Fe^{++}-activation broadens the specificity range so that different types of proteins can be attacked.

2. A 2-enzyme system may be present, as once postulated for papain by Bergmann [1937]. In this case the initial activity would represent a proteinase I, which would not be influenced by SH-Fe^{++}. A second enzyme, proteinase II, would be inactive under normal conditions, and active only in the presence of

heavy metal-SH complexes, and could be perhaps a type of peptidase. Attempts are being made to decide between the two ideas. Preliminary results indicate that the first theory is probably correct.

Recently two reviews of previous work on the subject of enzymic adaptation in micro-organisms were presented by Yudkin [1938] and Karstroem [1938]. Both authors distinguish between "adaptive" and "constitutive" enzymes. Quastel [1937] holds the view that the so-called constitutive and adaptive enzymes do not represent entirely different classes of enzymes. They represent rather the limits of variability of enzymes in a cell, the constitutive having the least range, and the adaptive the greatest range, of variability under different environmental and nutritional conditions. Our own view goes still further: we are inclined to picture the differentiation between constitutive and adaptive enzymes as a function of time; the longer a micro-organism is trained under altered environmental or nutritional conditions, the more will the properties of the constitutive enzymes disappear, until finally complete adaptation occurs. The many examples of the dissociation of various bacterial strains indicate that this view is probably not far wrong.

Yudkin [1938] has developed an interesting "mass action theory" of enzyme formation in bacteria. He postulates the existence of various inactive enzyme-precursors in the bacterial cell, and explains enzymic adaptation not by the formation of new enzyme, but rather by the conversion of the already existing precursor into active enzyme. The addition of substrate results in shifting the equilibrium of the enzyme-precursor system in the direction of active enzyme formation. On this basis, the results presented in Fig. 1, on the shift in the *p*H optimum of the secreted proteinases, would have to be explained as follows: the cultivation of *Cl. histolyticum* on gelatin at a low *p*H results in the secretion of a proteinase, already present in the cell in the precursor form, which attacks gelatin optimally at an acid reaction; cultivation in neutral gelatin results in the secretion of a different enzyme, also present in inactive form, which splits gelatin optimally at neutral reaction. Such an assumption is difficult to justify. The only difference in the stimulations to which the bacterial cells are subjected in the two cases is the difference in the *p*H of the media; the substrate remains the same. A more reasonable theory appears to be that recently proposed by Quastel [1937] which treats the enzymes as "cell metabolites whose rates of formation and destruction follow the same physicochemical laws as those controlling the metabolism of any other metabolite of the cell".

The action of an enzyme depends essentially on its active group. On the basis of known facts, it may be tentatively suggested that perhaps not every enzyme present in the bacterial cell has its own characteristic colloidal carrier. It may be that only a limited number of different colloidal carriers are present, which are interchanged as necessity demands, and according to definite laws, among the active enzyme groups already present or formed during bacterial growth. The extent of substrate specificity can of course be influenced also by the more detailed structural characteristics of the colloidal carrier. The views of Quastel and Yudkin may perhaps be brought into harmony as follows: the "enzyme-precursor" postulated by Yudkin may be identical with the colloidal carrier which is in equilibrium with the various prosthetic groups; through various external influences these equilibria within the cell are disturbed, so that on the basis of the mass action law a coalition between colloidal carrier and active groups occurs, resulting in the formation of a new enzyme.

SUMMARY

Several examples of enzymic adaptation in the proteinase of *Cl. histolyticum* are described. By changing the *p*H of the culture medium it is possible to change the *p*H optimum of the secreted proteinase. By repeatedly subculturing the bacteria on gelatin or casein media, enzymes are finally obtained which hydrolyse casein but not gelatin and vice versa. The mechanism of bacterial enzyme formation is discussed.

The authors wish to express their gratitude to Dr Ellice McDonald, Director, for his interest and support during this work.

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CCXXI. THE PREPARATION AND PROPERTIES OF A HIGHLY ACTIVE CATALASE FROM HORSE LIVER

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THE enzyme catalase has been investigated by a great number of workers. Zeile & Hellström's [1930] method of preparing horse liver catalase has been used with some slight modifications by most investigators. The most active preparation so far obtained is that of v. Euler & Josephson [1927] (Kat. f. 43,000). Sumner & Dounce [1937] have recently described a different method for the preparation of catalase from beef liver. Sumner's crystalline catalase showed the activity 28,000.[1] The present paper reports the preparation of an enzyme from horse liver, with an activity of 55,000–60,000.

The method of preparation

2 kg. horse liver are ground and extracted twice with 2 l. water for 6 hr. Haemoglobin and other inert proteins are precipitated with alcohol and chloroform according to Tsuchihashi [1923]. After centrifuging, the clear supernatant solution is evaporated at a low temperature down to 2 l. and mixed with 3 l. saturated ammonium sulphate solution. The precipitate is dissolved in 1·5 l. water after which an equal vol. of 95 % alcohol is added. Precipitated protein and salts are centrifuged down and 700 ml. alcohol more are added. The enzyme is precipitated and is separated from the solution after which it is dissolved in 1 l. water. 800 ml. saturated ammonium sulphate are added again. A compound with a high Fe-content (about 16 %) is thrown down. This compound accompanies the catalase, when preparations are made according to previously described adsorption methods, and is the cause of the widely different values reported for the iron content. This iron is easily split off and after acid hydrolysis gives the characteristic red colour with NH_4CNS. The ammonium sulphate concentration is raised to 58–60 % and the enzyme precipitate is filtered off. It is dissolved in water, reprecipitated with alcohol, and the preparation is then dialysed. The activity is 40,000–43,000. The preparation is stable for months at 0°. The yield is 0·8 g.

The preparation is further purified by adsorption on tricalcium phosphate. The diluted solution of enzyme (0·2 mg./ml.) is adjusted to about *p*H 5·5 by addition of KH_2PO_4 and is then filtered through a column of tricalcium phosphate as is usual with chromatographic analysis. The enzyme is adsorbed in a distinct layer at the top of the column and then eluted by a phosphate buffer at *p*H 8. The catalase solution is dialysed in cellophane bags.

[1] During Prof. Sumner's stay in Uppsala last winter I made a joint determination with him of Kat. f. of catalase, in the course of which we confirmed that our procedures were identical. The present values of Kat. f. are therefore directly comparable with those of Sumner & Dounce.

The activity of the preparation is 55,000–60,000. This procedure has been repeated a number of times with the same result.

The analyses show that the preparation contains 15·5% N (by micro-Kjeldahl) 0·085±0·005% Fe (by the method of Jorpes), and 0·02–0·03% Cu [by the [1933] method of Eisler *et al.* 1936].

For the determination of the activity the method described by v. Euler & Josephson [1927] was used. Kat. f. $= \frac{\text{reaction constant}}{\text{weight of preparation in g.}}$.

Determination of the spectrum

The light absorption at different wave-lengths was estimated by a photo-electric apparatus like that described by Warburg & Negelein [1929].

Stern [1937] has recently reported a quantitative determination of a catalase spectrum in the visible range. His preparation does not seem, however, to have been highly purified.

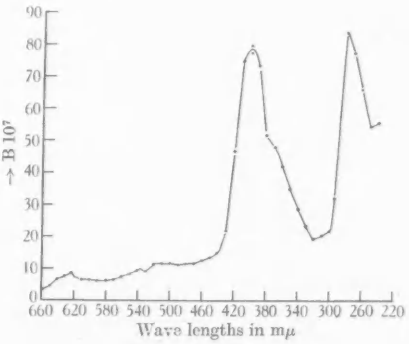

Fig. 1.

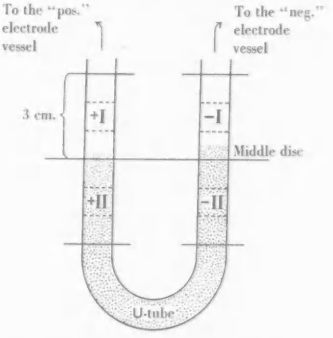

Fig. 2. The approximate position of the coloured preparation after the cataphoresis.

The molecular absorption coefficients are calculated from $\beta = \frac{1}{d}\frac{1}{c}\ln\frac{I_0}{I}$.

d = the depth of the cell in cm.

$c = \frac{\text{g. preparation}}{\text{ml.} \times \text{mol. weight}}$.

I_0 represents the intensity of the incident light and I the intensity of the transmitted light.

Table I. *The molar coefficients at the maxima of absorption*

Wave-length mμ	Molar absorption coefficients
623	$8{\cdot}3 \times 10^7$
536	$9{\cdot}4 \times 10^7$
400	$78{\cdot}5 \times 10^7$
280	$85{\cdot}0 \times 10^7$

Cataphoresis experiments

The purity of the preparation was tested with the cataphoresis apparatus of Theorell [1934]. The boundaries between the catalase solution and the buffer solution were level with the middle disk at the start of the experiment. During

the experiment the preparation migrated towards the anode. The displacement was counteracted by transferring the liquid from one vessel to the other, according to the principle introduced by Tiselius [1937]. In this way it was possible to have the boundaries of the enzyme solution at the end of the experiment approximately in the same position as at the beginning.

Conditions: *p*H 6·99. Conductivity $1{\cdot}37 \times 10^{-3}$. Current 5 mA. Time: 22 hr. and 40 min. Temp. 20°

8×2 ml. were brought from the "negative" electrode vessel to the "positive", corresponding to a displacement of the free boundaries of 8 cm.

The results of the analyses from the experiments are given in Table II.

Table II

		mg. prep./ml.	Kat. f.	% N	$10^{-2}k$/ml. N mg./ml.	% Fe	$10^{-1}k$/ml. Fe µg./ml.	% Cu	$10^{-2}k$/ml. Cu µg./ml.
The solution contained in the three cells above the middle disk	+I	2·0	50,000	14·9	3·30	0·1	7·8	—	—
Ditto below the middle disk	−I	5·0	51,000	15·5	3·47	0·082	6·6	0·044	1·15
,,	+II	9·55	54,000	15·8	3·35	0·091	5·8	0·027	1·9
,,	−II	11·4	52,000	15·3	3·41	0·079	6·2	0·026	2·0
The preparation in the U-tube		11·5	51,000	15·9	3·21	0·081	6·3	0·026	1·7
Preparation not submitted to cataphoresis		12·5	56,000	15·7	3·62	0·082	6·8	0·023	2·4

The contents of nitrogen and iron were both proportional to the activity of the solution in the cells. The copper also accompanied the activity, but in one cell (−I) the percentage was double as much as that usually found.

Ultracentrifuge experiments

Thanks are due to Prof. Svedberg for the opportunity of performing ultracentrifuge experiments at his Institute in co-operation with Nils Gralén. Using the light absorption method and light of wave-length 546 mμ the sedimentation constant of the iron-porphyrin-protein was found to be $11{\cdot}2 \times 10^{-13}$. The preparation proved however not to be homogeneous. Ultraviolet light photographs, 250–290 mμ, showed the presence of a component with a sedimentation constant of about $3{\cdot}2 \times 10^{-13}$. From dry weight determinations of the content in the two compartments of a "separation cell" [Tiselius *et al.* 1937] the concentration of this component is calculated to be 15–20 % of the total protein.

The diffusion constant was measured by the method of Lamm & Polson [1936] and was found to be $4{\cdot}3 \times 10^{-7}$ while the partial specific volume was determined as 0·715. Using the formula $M = \frac{RT\,s_{20}}{D_{20}(1 - V\zeta)}$ the molecular weight is calculated to be 225,000.

Stern & Wyckoff [1938] found the value 11×10^{-13} for the sedimentation constant of horse liver catalase.

Sumner & Gralén [1938] have found the sedimentation constant[1] for crystalline catalase from beef liver to be $11{\cdot}3 \times 10^{-13}$ and the diffusion constant $4{\cdot}1 \times 10^{-7}$, which give a value of $M = 248{,}000$.

Separation of a copper-protein from the iron-porphyrin-protein

The preparation (2 mg./ml. in $M/15$ Na_2HPO_4) was mixed with 1/3 of its volume of a saturated solution of picric acid. Then $N/10$ acetic acid was added till a precipitate appeared at *p*H 5·2. This was centrifuged down and more

[1] Corrected values, personal communication.

acetic acid added to the solution to bring the *p*H to 4·6, at which another fraction precipitated. These two fractions were dissolved separately in *M*/15 Na_2HPO_4 and were dialysed free from picric acid.

Each fraction has separately a considerably lower activity than the preparation before separation, i.e. fraction 1: Kat. f. 6000 and fraction 2: Kat. f. 3500. If however the original preparation is mixed with an equal volume of picric acid and the precipitation carried out by the addition of enough acetic acid to precipitate both fractions together at *p*H 4·6, the preparation retains the greater part of its activity after removal of the picric acid. This makes it probable that the inactivation is not due to the slightly acid reaction or to the action of the picric acid. It is possible therefore that both components may be necessary for the catalase activity.

The fraction which is first precipitated has an Fe content about 0·1 % and almost no Cu, whilst the other fraction has a Cu content of 0·16 % and very little Fe.

The sedimentation constants of the two isolated components have been determined. The Fe-porphyrin-protein had a sedimentation constant of about 12×10^{-13} and that of the Cu-protein was $3{\cdot}27 \times 10^{-13}$.

It is probable that the preparation by picric acid precipitation gives rise to the same two fractions which were observed in the earlier ultracentrifuge experiments, mentioned above.

Kubowitz [1937] has recently shown that an oxidative enzyme, polyphenoloxidase, is a Cu-protein (0·165 %). Keilin & Hartree [1938] have shown that the Fe in catalase is reduced by hydrogen peroxide from the trivalent to the bivalent state. It is however immediately oxidized in the presence of molecular oxygen. It seems possible, that the Cu-protein in this highly active catalase preparation might—by analogy with Kubowitz' oxidative enzyme—be necessary for the reoxidation of the bivalent Fe of the reduced catalase. The Cu-protein would in such a case be an integral part of a catalase system.

The activity was not, however, restored by a simple mixing of the two fractions nor was it raised by the addition of the Cu-protein to the original preparation. Further studies will however show, whether it is possible to separate the original preparation into two inactive fractions by other means than addition of picric acid and whether, in such a case the activity will be restored after mixing.

The differences in the activity of the preparation of Sumner & Dounce and that here described may be due not to impurities but to a difference in the structure of the protein (the preparations are made from different species of animal) or, if the Cu-protein is significant in the activity, to a different content of this substance. No information is given by Sumner & Dounce concerning the presence of copper.

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CCXXII. THE DISINTEGRATION OF TOBACCO MOSAIC VIRUS PREPARATIONS WITH SODIUM DODECYL SULPHATE

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It is well known that many bacteria are lysed and that toxins and antigens are modified by treatment with surface-active materials such as soaps and bile salts. Recently the commercially available wetting and spreading agents have been used in this way. Dean has summarized the chemical nature of these substances [1937]. The inactivation of diphtheria toxin by lauryl sulphate and the lysis of Gram-negative organisms have been studied by Bayliss [1936, 1937], and other workers, continuing the work of Larson *et al.* [1925], have prepared bacterial antigens by incubating suspensions of bacteria with these substances. This technique probably has no general application, for we have found that many large molecules are destroyed under these conditions. The destruction of preparations of potato virus "*X*" and of bushy stunt virus by incubation with the sodium salt of the sulphuric half ester of dodecyl alcohol has already been described [Bawden & Pirie, 1937; 1938, 1], and in this paper some observations on the disintegration of tobacco mosaic virus preparations will be reported.

Of this group of substances sodium dodecyl sulphate (Imperial Chemical Industries) and the apparently similar product called "Sulphonated" Lorol (Ronsheim and Moore) are the most efficient virus-inactivating agents that have been tested but the corresponding esters of cetyl alcohol and of the alcohols derived from palm oil, and the related amide, Igepon T, have some action; only sodium dodecyl sulphate (called SDS in future) has been studied in any detail.

The rate of action depends on temperature, concentration of SDS and *p*H. When tobacco mosaic virus preparations are disintegrated by SDS they become more acid, as they do when boiled [Bawden & Pirie, 1937]; it is therefore necessary to add a buffer to the system. Buffer solutions containing phthalates, phosphates or borates cannot be used, for even when dilute these form insoluble crystalline complexes with SDS. These complexes have not been investigated further. Veronal does not apparently combine with SDS and this buffer system [Michaelis, 1930] has been used. The action proceeds very slowly if at all in fluids more acid than *p*H 7·0; at *p*H 8·0 disintegration takes place and this *p*H value has been used in most experiments for in more alkaline solutions, although the action proceeds faster, there is greater spontaneous breakdown of the virus preparation.

The course of the action may be followed in five different ways, for on disintegration tobacco mosaic preparations lose their infectivity, serological activity, anisotropy of flow and sedimentability in a high-speed centrifuge and phosphorus and carbohydrate are no longer found in the precipitate which separates when the solution is one-third saturated with ammonium sulphate. An experiment in which the loss of infectivity and serological activity of a potato virus "*X*" preparation were followed has already been published [Bawden & Pirie, 1938, 1] and substantially similar results have been obtained with tobacco mosaic virus.

In this work a good preparation of virus, made by the method already described [Bawden & Pirie, 1937], was used. This preparation was colourless and a 2% neutral solution was nearly clear and wholly in the liquid crystalline state; it gave specific precipitation with antisera at a dilution of 1 : 8,000,000 and contained 0·5% phosphorus and 2·5% carbohydrate. The protein shows strong anisotropy of flow when a 1% solution is made in $M/20$ veronal buffer at pH 8·0; this solution gives an immediate precipitate when mixed with an equal volume of a 1% solution of SDS in buffer. The precipitate has the sheen and "crystalline", or more correctly fibrous [Bernal & Fankuchen, 1937], appearance under the microscope that characterizes the precipitates given by tobacco mosaic virus with acids, strong salt solutions or clupein. After about $\frac{1}{2}$ hr. at room temperature or after a shorter time at 37° the precipitate dissolves and the anisotropy of flow can now be followed by shaking the mixture in a tube about 1 cm. wide between crossed Nicols. The intensity of the anisotropy of flow falls off rapidly and after about 2 hr. at 37° it is barely perceptible, i.e. it corresponds to that given by a 0·04% solution of the original preparation. The anisotropy of flow disappears completely after about 10 hr. incubation. Nothing can be sedimented from a solution treated in this way by centrifuging for 3 hr. at 14,000 r.p.m. (17,000 times gravity). If the incubation is stopped before the anisotropy of flow has disappeared completely a small birefringent pellet with the properties of tobacco mosaic virus is sedimented and material can be isolated, which contains carbohydrate and phosphorus and which precipitates specifically with tobacco mosaic antisera.

If samples of a buffered mixture containing 0·5% of both virus preparation and SDS are withdrawn after various periods of incubation and added to 2 vol. of $\frac{1}{2}$ saturated ammonium sulphate solution containing enough ammonia to make the solution alkaline to phenol red, it is found that the amounts of carbohydrate and phosphorus in the supernatant fluid after centrifuging increase as the anisotropy of flow disappears and that the ammonium sulphate precipitate is free from nucleic acid when it separates from a solution giving no anisotropy of flow. This shows that in addition to inactivating the virus and breaking down the protein of high molecular weight the SDS separates the nucleic acid from the protein to which it was attached.

The action, as measured by any of these methods, is very greatly affected by changes in the concentration of SDS and in the ratio of its concentration to that of the virus preparation. If the concentration of SDS is doubled the disruption of the virus is complete in 1 hr. at 37°, whereas if the concentration is halved the anisotropy of flow does not disappear completely even after many days if the initial virus concentration is 0·5%, but if the virus concentration is reduced to 0·2% the anisotropy of flow disappears in the usual way.

To prepare a quantity of the nucleic acid-free protein 50 ml. of a 1% solution of virus preparation are mixed with 15 ml. of 5% SDS. It is unnecessary to buffer the mixture but $N/10$ NaOH is added from time to time to keep the pH between 8·0 and 8·5. After incubation for 24 hr. at 37° the mixture is filtered on a grade 4·5 Bechhold membrane (Schleicher & Schull). Most, but by no means all, of the SDS is found in the filtrate. The filter residue is dissolved in 5–10 ml. of water and dialysed thoroughly in a cellophane tube; this is necessary for the SDS is easily precipitated by ammonium sulphate. After a few days the dialysis sac contents no longer froth strongly. The pH of the fluid is now adjusted to 8–9 and saturated ammonium sulphate solution is added; with about 1/10 of a volume there is copious precipitation and on centrifuging the precipitate separates as a clear, gelatinous, coherent mass; this is redissolved and repreci-

pitated in the same way. If the disruption of the virus preparation has been complete there will be no further precipitation on the addition of more ammonium sulphate but if any protein remains undisrupted it will be precipitated. Nucleic acid precipitates from the supernatant fluid on acidification; if however the ammonium sulphate precipitation is carried out under slightly acid conditions part of this nucleic acid will precipitate with the protein.

The nucleic acid-free protein may be precipitated many times with ammonium sulphate but it is found that this treatment converts it in part into an insoluble material and in part into protein that is very easily precipitated by ammonium sulphate. After four or five reprecipitations it precipitates with 1–2 % solutions of ammonium sulphate and of certain other salts. This apparent denaturation also takes place if the neutral protein is kept at room temperature for some days and even more rapidly if it is made slightly acid. The insoluble protein, like the denatured protein made from tobacco mosaic virus preparations in other ways, is readily hydrolysed by trypsin preparations, whereas the protein prepared from disrupted virus by one or two precipitations with ammonium sulphate resembles "native" proteins in its resistance to proteolysis. It would appear that the products of disruption contain undenatured protein in an unstable state, they therefore resemble in many ways the "metaprotein" prepared by Theorell [1937] as a result of the removal of the lactoflavinphosphoric acid from Warburg's "yellow enzyme". In each case the protein which is made by splitting the starting material into a protein and a non-protein part is less stable than the intact molecule, but the unstable protein from tobacco mosaic virus can be kept at 0° for some weeks without apparent change. If however solutions which contain both protein and nucleic acid, but from which the SDS has been removed by dialysis, are kept, a gelatinous or slimy precipitate separates containing both protein and nucleic acid and only soluble if the *p*H of the fluid is raised above 8·5. The nucleic acid-free protein gives all the usual protein colour reactions and precipitates with the usual protein precipitants; its absorption spectrum resembles closely that of other proteins, i.e. there is an absorption maximum at 2750–2800 Å. and general absorption below 2500 Å. This supports the view that the intense absorption maximum at 2600 Å. which is found in tobacco mosaic and bushy stunt virus preparations [Bawden & Pirie, 1938, 2] is due to nucleic acid, for this absorption maximum is absent from the nucleic acid-free protein preparations.

Many attempts at fractionation have been made using acid, ammonium sulphate and similar agents but so far it has not been found to be heterogeneous if the incubation with SDS has been continued for a time sufficient to inactivate the virus completely. This observation sheds some light on the constitution of the protein of high molecular weight, which is the only recognizable component of a fully active virus preparation, for it seems to disintegrate into a nucleic acid and a protein that is too large to pass through a cellophane membrane but too small to sediment in a few hours in a centrifugal field of 17,000 times gravity. Bernal & Fankuchen [1937] have stated that tobacco mosaic virus preparations have not been made to give true crystals *in vitro* but that the submicroscopic rods which are responsible for the physical properties of virus preparations have an internal regularity which might be described as crystalline. It has been suggested [Bawden & Pirie, 1937] that these rods are artefacts made by the irreversible linear aggregation of the virus particles as they occur in the sap of young plants. Study of the disruption of tobacco mosaic virus preparations suggests that these units (which have as yet only been studied by filtration methods), from which the rod-shaped aggregates are built up, are themselves built from a number of

similar or identical proteins of normal molecular weight. The methods that have been used so far for studying these products are however too crude to give a definite result. Some attempts have been made to prepare antisera using the disrupted virus as an antigen but these have been unsuccessful.

Summary

The kinetics of the disintegration of a tobacco mosaic virus preparation by sodium dodecyl sulphate are described and also the preparation and properties of the resulting unstable protein.

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CCXXIII. PYRUVATE OXIDATION IN BRAIN
IV. THE OXIDATION PRODUCTS OF PYRUVIC ACID

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Detailed study of the biological oxidation of pyruvic acid is desirable owing to its important role as an intermediary metabolite in carbohydrate breakdown by tissue. A summary and discussion of some earlier work on pyruvic acid metabolism has been given in previous papers from this laboratory [Peters, 1936; 1937; McGowan, 1937].

Recently, several quantitative investigations have been made. Working on slices of rat brain, Weil-Malherbe [1937, 2] showed that under anaerobic conditions the amounts of lactic acid and CO_2 formed from pyruvic acid were quantitatively given by the Krebs dismutation [Krebs & Johnson, 1937, 1]:

$$2CH_3CO.COOH + H_2O \rightarrow CH_3CHOH.COOH + CH_3COOH + CO_2.$$

Only 80% of the acetic acid was found, but the deficit could be accounted for approximately by formation of succinic acid. With brei, the tendency was to form more succinic acid at the expense of acetic acid.

In aerobic studies, McGowan [1937] determined the O_2/pyruvic acid ratio for pigeon brain brei, obtaining a value of 450 μl./mg., the theoretical for complete combustion being 635. This evidence of incomplete oxidation was confirmed by the high value found for the respiratory quotient, 1·3, as compared with the calculated value of 1·2 for complete oxidation. It was also shown that under these conditions the formation of a small amount of lactic acid accompanied the respiration, but not sufficient to account for the low value of the O_2/pyruvate ratio, assuming the lactic acid to arise from the Krebs dismutation.

The object of the experiments to be described was to continue the above work, which involves determining the amounts of lactic and acetic acids formed during the respiration, and finally to obtain a complete balance sheet for the oxidation of pyruvic acid under these conditions.

Experimental method

The procedure differs in one important respect from that of McGowan, namely that unwashed tissue has been used in all cases. The reason for this is that the pyruvate oxidase system in brain is to some extent destroyed by washing, with consequent fall in respiration, thus making difficult the accurate determination of the small quantities of lactic and acetic acids involved. This difference in technique leads to slight corrections in the increased O_2 uptake due to pyruvate. The appropriate corrections to be applied are dealt with separately in the sections dealing with the calculation of lactic and acetic acid formation.

The brain was prepared in the following way. Three pigeons were killed by decapitation and the brains dissected out. The membranes and cerebellum were removed and the rest thoroughly minced with a bone spatula on a warm (38°) plate. The brei was then transferred, in roughly equal quantities (350 mg.), to the experimental bottles.

Na pyruvate[1] was used as substrate. A pure sample of this was kindly supplied by Prof. R. A. Peters. A fresh solution in *M*/10 phosphate Ringer solution, *p*H 7·3, was made up for each experiment, such that 0·2 ml. contained about 10 mg. This amount was added to each bottle containing a total of 2·8 ml. fluid; final concentration *c.* 0·03 *M*.

The respiration was measured as follows. The brain brei was introduced into the previously weighed bottles, each containing 1·5 ml. Ringer phosphate, *p*H 7·3, as used in previous experiments in this laboratory. After reweighing, the tissue was divided with a glass rod, 0·2 ml. of the Na pyruvate solution added and the total volume, including tissue, made up to 2·8 ml. with Ringer phosphate. In each experiment, four series of apparatus were used:

(1) Containing only tissue, stopped at the end of the equilibration period, i.e. just before the measurement of the respiration.

(2) Containing only tissue, not stopped until the end of the respiration.

(3) Containing tissue and pyruvate, stopped at the end of the equilibration period.

(4) Containing tissue and pyruvate, stopped at the end of the respiration.

Respiration was measured in O_2, using Barcroft-Dixon manometers. The usual technique of this laboratory was employed for the absorption of CO_2 (filter paper with 2*N* KOH). The rate of shaking was 108 oscillations per min. the bath being kept at 38°.

Determination of the lactate/pyruvate ratio

The difference in lactate content of bottles 3 and 4 gives the amount of lactate formed from pyruvate during the respiration period. The difference in O_2 uptake of apparatus 2 and 4 gives the uncorrected O_2 uptake associated with the formation of this amount of lactate. At the beginning of the respiration period, the amount of residual lactate is about 0·6 mg. per g. tissue, and this falls to about 0·2 mg./g. at the end of the respiration (Table I).

Table I. *Residual lactic acid*

	mg. per g. tissue		
Exp.	Initial	Final	Difference
90	0·56	0·20	0·36
92	0·57	0·16	0·41
93	0·69	0·29	0·40
Average	0·61	0·22	0·39

This residual lactate disappearing is given by the difference between the lactate contents of bottles 1 and 2. The O_2 uptake associated with the oxidation of this 0·39 mg. lactic acid to pyruvic acid and beyond is 222 μl. In bottles 3 and 4, the presence of pyruvate inhibits this lactate oxidation [Green & Brosteaux, 1936] with its corresponding O_2 uptake. Hence the observed increased O_2 uptake, due to disappearance of pyruvate, must be corrected by adding 222 μl. per g. tissue. From this corrected O_2 uptake the amount of pyruvate disappearing during the respiration has been calculated, using the value for the ratio O_2 uptake/pyruvate disappearing = 450 found by McGowan [1937]. In these experiments, the respiration was usually measured in duplicate or triplicate. The initial values quoted in Table I for residual lactate must not be confused with those observed by Kinnersley & Peters [1929; 1930] for lactate

[1] We are greatly indebted to Dr E. Stedman for recommending the use of Na pyruvate.

formed in brain soon after death. Amounts of the order of 1 mg. lactate per g. brain were formed from carbohydrate within about 1 min. after decapitation. Those in Table I are found roughly 90 min. after mashing, the smaller values being due presumably to oxidation of lactate to pyruvate in this system.

The estimation of lactate in the Barcroft bottles was carried out in the following way. Each bottle was treated with 1 ml. 25% trichloroacetic acid to precipitate the proteins and prevent further respiration, and after ½ hr. the contents were filtered. The bottle was then washed out twice with 2 ml. 5% trichloroacetic acid. To the combined filtrates were added 5 ml. 10% $CuSO_4$ and 6 ml. of a 10% $Ca(OH)_2$ suspension. After ½ hr. the volume was made up to 25 ml. and the bulky blue precipitate centrifuged off. 15 ml. of the centrifugate were used for each lactate estimation, according to the method of Friedemann & Kendall [1929], using the improved absorption tower of R. B. Fisher (private communication) and Na_2HPO_4 for aldehyde liberation [Lehnartz, 1928]. The average recovery of added lactate, using pure Zn lactate, was 98%.

Table II summarizes the results of these experiments. In every case the value of the ratio lies between 4·9 and 5·4, the variations being within the experimental error of the method. The average value is 5·2 mol. lactic acid per 100 mol. pyruvic acid.

Table II. *Lactic acid formation*

Exp.	Duration in min.	Tissue mg.	O_2 uptake (μl.) Uncorr.	Corr.	Pyruvic acid mg. (calc.)	Lactic acid mg.	L.A./P.A. molar %
91	210	404	1901	1990	4·42	0·25	5·4
92	180	366	1557	1635	3·63	0·20	5·4
		343	1452	1531	3·40	0·19	5·4
		339	1444	1517	3·37	0·18	5·1
93	200	355	1581	1660	3·69	0·19	4·9
		362	1611	1691	3·76	0·20	5·1
94	195	283	1203	1267	2·82	0·15	5·3
		307	1305	1373	3·05	0·16	5·2

Determination of the acetate/pyruvate ratio

As in the determination of lactate, the acetate production from respiration in pyruvate is equal to the difference in acetate content of bottles 3 and 4. There is a small amount of residual acetate formation, which is given by subtracting the acetate formed in 1 from that in 2. The net acetate production is therefore the difference between these two values. The small correction to be applied to the O_2 uptake is almost negligible, being only the O_2 absorbed in converting 0·39 mg. lactate into pyruvate, a value of about 50 μl. per g. tissue.

The estimation of acetate was based on the distillation method used by Weil-Malherbe [1937, 1, 2]. There are several substances which are volatile under the greatly reduced pressure employed. Free pyruvic acid, which is fairly volatile, is eliminated by conducting the distillation in the presence of phenylhydrazine [Weil-Malherbe, 1937, 2]: addition of pyruvic acid to the distilling flask did not increase the acidity of the distillate. Lactic acid is also slightly volatile under these conditions, but the amount distilled does not increase in proportion to its concentration [Virtanen & Pulkki, 1928]. In order to counteract any effect due to varying low concentrations, all distillations were carried out in the presence of a large excess of lactic acid, so that it was accounted for in the

blank estimation. The contents of the bulb of the Parnas apparatus were kept at about *p*H 2, at which reaction HCl is not liberated.

Each bottle was treated with 0·2 ml. 10% H_2SO_4 to precipitate protein and prevent further respiration. There was evidence that the use of sulphuric acid for this purpose did not affect the results, since similar values were obtained, in another connexion, when further respiration was prevented merely by placing the bottles in cold-store. When precipitation was complete—the bottles were usually allowed to stand overnight in the refrigerator—the contents were centrifuged, 2 ml. of the clear liquid being used for each estimation. The total amount of acetate was calculated from the volume of fluid in each bottle, tissue being counted as fluid volume, since the amount of solid matter is only about 15% of the wet wt. of the brain.

To the sample were added 10 ml. of a solution containing 1 ml. 10% phenylhydrazine hydrochloride, 1 ml. syrupy phosphoric acid and 5 g. NaH_2PO_4. 0·2 ml. (10 mg.) lactic acid was added. The mixture was allowed to stand for at least ½ hr. before distillation, distilled water (2 ml.) being used to wash it completely into the bulb of the Parnas apparatus. Distillation in steam was carried out at 14–16 mm. pressure, an exact amount of distillate (55 ml.) being collected in each case. This was then titrated against freshly prepared *N*/200 CO_2-free NaOH, using bromothymol blue as indicator. The standard colour tint was a solution of sodium acetate to which a few drops of the indicator had been added. A clearly defined end-point at *p*H 7·6, sharper than with phenolphthalein, was obtained. Blank estimations were always performed, the usual value being about 0·25 ml. *N*/200 NaOH. Under these conditions, the recovery of added acetic acid, as shown in Table III, was 87·3%. Higher recoveries could be obtained by collecting more distillate, but this proved inconvenient.

Table III. *Recovery of added acetic acid*

Acetic acid mg.	*N*/200 NaOH (ml.) Calc.	Found	Recovery %
0·650	2·166	1·898	87·6
		1·884	87·0
0·325	1·083	0·979	90·4
		0·972	89·8
0·163	0·542	0·469	86·6
		0·457	84·4
0·065	0·217	0·185	85·3

The procedure therefore differs from that of Weil-Malherbe in the following respects. Enzymic oxidation of pyruvic acid was arrested by the use of 10% H_2SO_4. A stock solution of NaH_2PO_4, phosphoric acid and phenylhydrazine hydrochloride—"acetate mixture"—was employed for all distillations, and proved of great convenience. All estimations were carried out in the presence of a large excess of lactic acid. Finally bromothymol blue was used as indicator.

Table IV. *Acetic acid formation*

Exp.	Duration in min.	Tissue mg.	O_2 uptake (μl.) Uncorr.	Corr.	P.A. mg. calc.	Acetic acid mg.	H.Ac./P.A. molar %
80	170	327	1131	1147	2·55	0·42	24·4
81	175	392	1432	1452	3·23	0·55	25·1
82	240	383	1895	1914	4·25	0·71	24·5
		368	1891	1908	4·24	0·74	25·5
86	185	356	1329	1346	2·99	0·53	25·9
		370	1367	1385	3·08	0·52	24·7
		377	1402	1421	3·16	0·51	23·8
87	185	348	1162	1180	2·62	0·44	24·6
		370	1217	1234	2·74	0·47	25·0

Acetic acid was identified by the method of Krüger & Tschirch [1929]. Four distillates were neutralized, evaporated to about 1 ml. and treated with 5 % lanthanum nitrate (0·5 ml.), $N/50$ I_2 in KI (0·5 ml.) and 1 drop of $4N$ NH_4OH. On warming gently at first and finally boiling, the solution turned green and then blue, a characteristic blue precipitate separating out on cooling. This identified either acetate or propionate. Since the formation of the latter was unlikely, acetate was inferred.

Table IV summarizes the results of the experiments. It will be seen that the % ratio lies between 23 and 26 in every case, with an average of 24·8.

Balance sheet of pyruvic acid disappearance

Since the amount of lactic acid formed is so very small, the fate of pyruvic acid cannot be accounted for merely by the summation of the amounts which are completely oxidized or which follow the anaerobic Krebs dismutation. It cannot be argued that the observed formation of lactic acid is too small owing to reoxidation to pyruvic acid. As previously stated, the presence of pyruvic acid should inhibit completely the lactic dehydrogenase. Moreover, the Krebs dismutation cannot account for such a large production of acetic acid. To the above two processes

I. $CH_3CO.COOH + \frac{5}{2}O_2 \rightarrow 2H_2O + 3CO_2$,
II. $CH_3CO.COOH + \frac{1}{2}H_2O \rightarrow \frac{1}{2}CH_3CHOH.COOH + \frac{1}{2}CH_3COOH + \frac{1}{2}CO_2$,

must be added a third

III. $CH_3CO.COOH + \frac{1}{2}O_2 \rightarrow CH_3COOH + CO_2$,

which will account for the excess of acetic acid formed over and above that produced by the Krebs dismutation.

The method of calculating the percentages of the above processes is as follows. 5·2 % lactic acid can only be derived from the Krebs dismutation; hence process II takes place to the extent of 10·4 %. Of the acetic acid formed, 5·2 % comes from the Krebs dismutation, so that the remaining 19·6 % is formed in process III. The amount of I can be deduced from a consideration of the O_2/pyruvate ratio.

450 μl. O_2 are required to oxidize 1 mg. pyruvic acid under these *in vitro* conditions. Process III oxidizes 1 mg. pyruvic acid with an accompanying 127 μl. O_2. Then, since this process takes place to the extent of only 19·6 %,

$$19{\cdot}6/100 \times 127 = 25\,\mu\text{l.}$$

of O_2 are absorbed. All the remaining O_2 is used up by process I. For complete oxidation, 1 mg. pyruvic acid requires 635 μl. O_2; it follows, then, that the amount undergoing complete oxidation is

$$425/635 \times 100 = 67\,\%.$$

The disappearance of pyruvic acid can thus be accounted for quantitatively by an appropriate summation of the three following equations. The extent to which each process takes place is represented by the accompanying percentages.

$CH_3CO.COOH + \frac{5}{2}O_2 \rightarrow 3CO_2 + 2H_2O$	67 %
$CH_3CO.COOH + \frac{1}{2}H_2O \rightarrow \frac{1}{2}CO_2 + \frac{1}{2}CH_3CHOH.COOH + \frac{1}{2}CH_3COOH$	10·4 %
$CH_3CO.COOH + \frac{1}{2}O_2 \rightarrow CO_2 + CH_3COOH$	19·6 %

97 % of the pyruvic acid is accounted for.

From the above data, the respiratory quotient can be calculated:

$$\text{R.Q.} = \frac{5{\cdot}2 + 19{\cdot}6 + 3\,(67)}{\frac{1}{2}\,(19{\cdot}6) + \frac{3}{2}\,(67)} = \frac{225{\cdot}8}{177{\cdot}3} = 1{\cdot}28.$$

This agrees well with the experimental value of 1·3 found by McGowan [1937]; in fact if his one high value is excluded his average R.Q. is 1·285.

Discussion

The preceding section provides us with a detailed knowledge of the ultimate fate of pyruvic acid when enzymically oxidized by pigeon brain. The importance of this knowledge is that we now have quantitative data which any theory of aerobic pyruvic acid oxidation must take into account. The results must, however, be interpreted with care.

The fact that a balance sheet can be drawn up by combining three equations in an appropriate manner does not necessarily mean that all three processes can take place independently of one another, or even that, as such, they take place at all. It may well be that the formation of lactic acid by the Krebs dismutation is independent of the oxidative processes. Nevertheless there is no direct evidence that lactic acid is formed in this manner in the system. It may possibly arise by some other process, so far unknown.

An "explosive" oxidation of pyruvic acid giving CO_2 and water, i.e. reaction I without any intermediary, seems most improbable. Several plausible theories of pyruvic acid oxidation have been proposed involving a series of oxidation products usually in the form of a cycle, as in the Toenniessen-Brinkmann [1930] scheme and the Krebs & Johnson [1937, 2] citric acid cycle. If there were no evidence to the contrary [McGowan & Peters, 1937], these might appear possible for aerobic oxidation in brain.

The possibility of acetic acid being an intermediate stage of pyruvic acid metabolism is very unlikely, since it has no noticeable effect on brain respiration, either in the presence or absence of pyruvate [McGowan & Peters, 1937; Elliott *et al.* 1937]. If the formation of acetic acid is not independent of other oxidative mechanisms, then it may conceivably arise from the breakdown of some initial stage of an unknown cycle. It is suggested that the following scheme might show the relationship between acetic acid formation and some general mechanism of pyruvate oxidation:

Pyruvic acid → (X) → Acetic acid

(X) → Cycle regenerating some pyruvic acid, CO_2 and water being eliminated.

Hence a cyclical theory must involve some unknown stage (X).

It is clear from the balance sheet that if there is any formation of citric acid [Simola & Alapeuso, 1938], then it could only arise from about 3 % of the pyruvic acid apparently unaccounted for. Of course, the above remarks apply only to aerobic pyruvate oxidation in brain, and do not in any way embrace anaerobic oxidations such as are brought about by methylene blue.

Summary

Lactic and acetic acids have been found in definite amounts when pigeon brain brei has respired in pyruvic acid. The quantities determined account for 30 % of the pyruvate disappearing, the remainder being oxidized completely to CO_2 and water, thus enabling a complete balance sheet to be drawn up.

I wish to express my thanks to the Rockefeller Foundation and to the Medical Research Council for grants to Prof. R. A. Peters in aid of this work. I am also greatly indebted to Prof. Peters for his keen interest, help and advice; to Mr H. W. Kinnersley for assistance with the pigeons; and to Mr R. W. Wakelin for occasional help.

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CCXXIV. THE EFFECT OF CERTAIN BACTERIAL TOXINS UPON SOME RESPIRATORY MECHANISMS OF ANIMAL TISSUES

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Little work has hitherto been done to ascertain the effect of bacterial toxins upon physiologically active enzyme systems. Walthard [1934] reported that the addition of diphtheria toxin in bouillon to a rat liver preparation in a Barcroft micro-respirometer exerted no action upon the rate of O_2 absorption. On the other hand, Cross & Holmes [1937] reported that the power of isolated liver tissue of rabbits to form extra carbohydrate in the presence of various substrates is much lower for tissue obtained from animals suffering from diphtheritic toxaemia than for tissue obtained from normal rabbits. They also showed that in the presence of pyruvate the rate of O_2 uptake of liver slices obtained from a toxaemic rabbit is subnormal.

In this paper experiments are reported in which various toxins obtained from members of the *Clostridium* group of bacteria have been added to suspensions of actively respiring tissues in a Barcroft differential micro-respirometer.

Experimental

Corbet & Wooldridge [1936] pointed out the necessity for replication[1] and repetition of experiments made with the Barcroft differential manometer, especially when comparisons are made between results which do not differ widely. To record a result merely as the average of so many experiments without giving the standard error makes the acceptance of such results difficult. Our own experience with tissue respiration studies reinforces us in our view that results are often misleading unless experiments be both replicated and repeated and the final results expressed statistically.

Most of the work described has been done with suspensions in tap water of small intestine minced in a Latapie mincer after washing to remove intestinal contents. Other tissues have been used and the technique of their preparation is similar, the tissues being minced in the Latapie apparatus (liver, kidney, skeletal muscle) or ground in a mortar (brain, heart muscle), suspended in tap water and filtered through muslin in order to obtain a more homogeneous suspension. Owing to the fact that the minced tissues actively oxidized succinate, and to the advisability for replication of results, which was relatively easy with suspensions of finely minced tissue, the tissue slice technique was not followed in this work.

The tissues were obtained from guinea-pigs, and were used within 1 hr. of the death of the animal. The small intestine was employed to a great extent as

[1] By "replication" is meant the setting up of a series of identical experiments at one and the same time.

Cl. welchii is frequently the cause of intestinal trouble in certain animals. In the manometric experiments tissue was placed in each cup, and usually the only difference between the contents of the left and right cups was the addition of substrate to the latter. Many substrates have been tried but mainly succinate has been used. The results given are based on experiments lasting 40 min. at 37°, in which the rate of O_2 absorption was still proceeding linearly. There was usually a diminution in the rate after 1 hr. The experiments were continued for 2 hr. to make sure that their course was normal. The majority of our experiments were put up in quadruplicate or higher replication and each experiment was usually repeated at least 5 times. In the tables the results are expressed as means, with the standard errors given in brackets. In order to facilitate statistical analysis, the mean of the replicated results from any particular experiment was expressed as 100 for the O_2 absorption in 40 min. by a substrate alone in the presence of a particular tissue but in the absence of toxin or antiserum. (The actual results varied in the different experiments between 80 μl. and 150 μl. for succinate.) The figures for the O_2 uptake in the presence of toxin or antiserum were altered in the same proportion to facilitate comparison.

The micro-respirometers contained air and were shaken at 37°, the readings being started after 10 min. temperature equilibration. A roll of filter paper soaked in KOH solution, to absorb CO_2, was present in the centre cup of all manometric flasks.

The toxins and antitoxins used were obtained through the kindness and generosity of Dr O'Brien, Prof. Dalling and Dr Montgomerie of the Wellcome Physiological Research Laboratories, Langley Court, Beckenham, Kent, England. These toxins were prepared from broth filtrates of cultures of the appropriate anaerobe by precipitation with 70% ammonium sulphate, the deposit being dried *in vacuo*. The broth used was made from horse muscle with the addition of Parke Davis peptone and a large proportion of cooked meat in the case of *Cl. welchii*, types A and D, and of Witte peptone with only a small amount of cooked meat in the case of the type C organism. As a control for these toxins a similar preparation was made from the uninoculated broth used for *Cl. welchii*, type A, and this is alluded to in the tables and the text as the broth precipitate or broth precipitate control. The strength of toxin usually used in the manometric flasks was 0·4 mg. dried material per flask. The tissues used were usually washed and suspended in tap water but the contents of the flasks were maintained at *p*H 7·4 by the addition of 0·5 ml. *M*/20 phosphate buffer. No change in *p*H occurred during the experiments. The average dry wt. of tissue (small intestine) added to the flasks was 14 mg., but it varied between 11 and 16 mg. and on occasion was as high as 20 mg. Where toxin and antiserum were used in the same cup they were mixed and allowed to stand for 15 min. before adding to the tissue.

Aerobic oxidations by minced small intestine. Krebs [1933; 1935] and others have reported no marked oxidative activity of small intestine with various substrates. Using aqueous suspensions of minced small intestine in the micro-respirometer we have been unable to show any definite oxidizing activity for any substrate other than succinate, *p*-phenylenediamine and dihydroxyphenylalanine (Table I). Under our experimental conditions we were unable to show any activity with glucose, fructose, sucrose, lactate, pyruvate, fumarate, malate, oxalate, butyrate, oxaloacetate, citrate, formate, glycerol, alanine, serine, tryptophan, glutamate, aspartate, phenylalanine, glycylglycine, leucylglycine, glycyltryptophan or glycyltyrosine.

Table I. *Aerobic oxidations by small intestine*

Experimental details in the text. Each experiment with succinate and with *p*-phenylenediamine was quadruplicated and with dihydroxyphenylalanine was triplicated. The *p*-phenylenediamine was used as the hydrochloride neutralized with NaOH. The mean values are given for the dry wt. of tissue used and for the "Q_{O_2}". The O_2 absorbed is due to the substrate in the presence of the tissue, the tissue being present in both cups, hence "Q_{O_2}" here represents the difference in true Q_{O_2} caused by substrate addition.

Substrate	No. of exps.	Dry wt. of tissue	Q_{O_2}
Succinate *M*/120	30	13·70	11·73 (S.E. = 0·47)
p-Phenylenediamine *M*/54	12	15·85	10·33 (S.E. = 0·20)
Dihydroxyphenylalanine *M*/120	5	16·78	3·65 (S.E. = 0·40)

The effects of various bacterial toxins upon the aerobic oxidation of succinate in the presence of small intestine. Various toxins obtained from certain anaerobic bacteria were added to mixtures of succinate and small intestine in Barcroft micro-respirometers, and their effects upon the rate of aerobic oxidation observed. It will be seen from Table II that all the toxins inhibit the oxidation to some small

Table II. *The effects of various bacterial toxins upon the aerobic oxidation of succinate in presence of small intestine*

The figures given for the O_2 uptake represent the means, and their standard errors, obtained from the numbers of experiments mentioned. Details in text. The meaning of the symbols used is as follows. S = *M*/120 succinate, BP = control broth precipitate, A, C and D represent the toxins obtained respectively from *Cl. welchii*, types A, C and D. Oed., Sept., and Tet. represent the toxins obtained respectively from *Cl. oedematiens*, *Cl. septique* and *Cl. tetani*. All toxins were used in a concentration of 0·4 mg. dried product per manometric cup. S.E. = standard error, and *P* might be called the probability of significance. If P^* <0·01 this is regarded as significant.

	S	S+BP	S+A	S+C	S+D	S+Oed.	S+Sept.	S+Tet.
No. of exps.	121	9	63	39	18	6	6	5
No. of manometers	467	27	237	141	69	23	24	18
Mean O_2 uptake	100·11	82·41	15·79	35·91	70·52	77·80	86·53	81·63
S.E.	0·46	1·04	0·35	0·71	1·56	1·69	2·35	2·15
Values for *P* compared with:								
(*a*) Succinate control	—	<0·01	<0·01	<0·01	<0·01	<0·01	<0·01	<0·01
(*b*) Broth ppt. control	—	—	<0·01	<0·01	<0·01	0·1–0·2	0·2	0·8–0·9

* For methods of statistical calculations see Fisher [1936].

extent but that a similar inhibition is induced by the "broth precipitate". Only the toxins of *Cl. welchii* produce inhibitions significantly greater than that of the broth precipitate, and of these inhibitions that induced by the type A organism is greatest and that by the type D least. The extent of the inhibition varies with the amount of toxin added, as is shown in Table III, but at the concentration chiefly employed in these particular studies, viz. 0·4 mg. toxin per cup, the inhibition by the toxins of the A, C and D types of organism are respectively 84·21, 64·09 and 29·48% (Table II). The proportion of the α, β and ϵ toxins present in these three toxins is given in Table III and it will be seen that the α toxin is present in all. The β toxin is only present in the toxin from the type C organism and the ϵ toxin only in that from type D, and, in comparison with α toxin, their concentrations are high. These results suggest, therefore, that the inhibition of the succinate oxidation may be due to the α toxin, but that, in any case the inhibitory actions of the β and ϵ toxins must be small.

Table III. *The effect of the toxins of* Cl. welchii *in different concentrations upon the aerobic oxidation of succinate by small intestine*

The upper part of the table indicates the nature of the toxin preparations used, and the lower half gives the % inhibition produced by the various toxins in the concentration indicated in the first column. Experiments were made in quadruplicate and the standard errors were small.

Toxin	Type A	Type C	Type D	Broth ppt. control
Units toxin per 100 mg.				
α	12·5	Some	Some	0
β	0	100	0	0
ϵ	0	0	800	0
	Inhibition of succinate oxidation (%)			
Mg. toxin per cup				
0·01	68	49	—	—
0·40	83	65	30	19
1·6	98	93	60	38
2·4	—	—	74	39

The effects of antisera upon the inhibition induced by the toxins of Cl. welchii. The oxidation of succinate by small intestine is inhibited by the presence of the toxins of *Cl. welchii*, but if various antisera are added as well as the toxin the inhibition induced by the toxin is considerably diminished by those antisera which contain antibodies for the α toxin. Table IV gives the results obtained

Table IV. *The effects of various antisera upon the inhibition by the toxin of* Cl. welchii, *type A, of the aerobic oxidation of succinate in the presence of small intestine*

The figures given in the table are the means, and standard errors, of five experiments each made in quadruplicate. The results have been adjusted so that the mean for the O_2 uptake due to succinate alone is 100. The α toxin present in the toxin used (0·4 mg. per cup) could be neutralized by a 1/10,000 concentration of antiserum or a 1/2800 concentration of the type D antiserum, as judged by mouse-inoculation experiments. S = succinate, AS = antiserum, N = normal horse serum, TX = toxin; other abbreviations as for Table II.

					% inhibition			% inacti-vation
Antiserum	S	S+AS	S+TX	S+TX +AS	TX	AS	(TX+AS) −AS	of TX
Welchii A, 1/10,000	100	88·70	15·00	47·60	85	11·3	41·10	51·7
S.E.	2·26	2·50	1·21	1·43				
Welchii C, 1/10,000	100·37	96·50	18·05	16·48	81·95	3·87	80·02	2·4
S.E.	2·23	2·28	1·71	1·35				
Welchii C, 1/4000	100·01	84·56	14·10	37·08	85·91	15·45	47·48	44·7
S.E.	1·37	1·61	0·80	1·41				
Welchii D, 1/10,000	100·65	92·95	17·85	16·20	82·80	7·70	76·75	7·3
S.E.	2·14	3·04	1·54	0·93				
Welchii D, 1/2800	100·25	85·22	13·55	49·92	86·70	15·03	35·30	59·3
S.E.	2·87	2·37	0·70	1·70				
Normal, 1/2800	100	91·90	11·20	8·40	88·80	8·10	83·50	6·0
S.E.	2·25	2·14	1·19	1·16				
Septique, 1/2800	100·25	88·55	13·65	9·40	86·60	11·70	79·15	8·6
S.E.	2·19	3·28	1·63	1·63				
Oedematiens, 1/2800	99·75	84·25	14·70	9·33	85·05	15·50	74·92	11·9
S.E.	1·84	2·44	1·09	1·10				
Tetani, 1/2800	100·27	85·10	13·85	8·35	86·42	15·15	76·75	11·2
S.E.	1·59	1·97	1·11	1·12				

with the toxin of *Cl. welchii* type A and various antisera, these sera being added in amounts equal to, or greater than, the concentration of the homologous antiserum which should neutralize the toxin, viz. 1/10,000. Of the antisera used only those for the three types of *Cl. welchii* contained antibodies for the α toxin. The antitoxic strengths of these three antisera were as follows: type A antiserum contained 300 units of α antitoxin per ml., type C antiserum contained 120 units of α antitoxin and 900 units of β antitoxin per ml. and type D antiserum contained 85 units of the α antitoxin and 275 units of the ϵ antitoxin per ml. From the results given in Table IV it is seen that when these three antitoxins are added in amounts equivalent in α antitoxin units they each reduce the inhibitory effect of the type A toxin by about half. The other antisera used and the normal serum produced no appreciable inactivation of the toxin. It will be noticed that all the sera themselves produced some small but significant inhibition of the succinate oxidation and this has been allowed for in calculating the extent of the

Table V. *The inactivation of the toxin of* Cl. welchii, *type A, by its homologous antiserum, as measured by the inhibition produced of the aerobic oxidation of succinate by small intestine*

Experimental details in the text. The figures given were obtained from replicated experiments and represent the O_2 absorbed.

Conc. of antitoxin	0	$\frac{1}{4} \times 10^{-4}$	$\frac{1}{2} \times 10^{-4}$	1×10^{-4}	2×10^{-4}	4×10^{-4}
No toxin	100	90	86	89	89	84
Toxin present	13	10	30	48	59	74

Table VI. *The effects of various antisera upon the inhibition by the toxins of* Cl. welchii, *type C and type D, of the aerobic oxidation of succinate in the presence of small intestine*

The figures given in the table are the means and standard errors of five experiments in the case of toxin C, and of fewer experiments in the case of toxin D, each made in quadruplicate. The results have been adjusted so that the mean for the O_2 uptake due to succinate alone is 100. The β toxin present in toxin C (0·4 mg. per cup) could be neutralized by a 1/3080 concentration of antiserum to the type C organism, while the ϵ toxin present in toxin D (1·6 mg. per cup) could be neutralized by a 1/66 concentration of antiserum to D (i.e. twice the actual concentration used), as judged by mouse inoculation experiments. S = succinate, AS = antiserum, Tx = toxin, N = normal horse serum and other abbreviations as for Table II.

					% inhibition			
Toxin + AS	S	S + AS	S + Tx	S + Tx + AS	Tx	AS	(Tx + As) − AS	% inactivation of toxin
Toxin C:								
Welchii A, 1/3080	100·20	81·95	30·92	48·35	69·28	18·25	33·60	51·5
S.E.	2·12	3·12	2·60	2·57				
Welchii C, 1/3080	100·11	85·46	37·99	51·26	62·12	14·65	34·20	43·95
S.E.	1·87	1·54	1·21	1·55				
Welchii D, 1/3080	100·32	82·58	30·42	39·01	69·89	17·73	43·58	37·7
S.E.	1·60	1·58	1·93	1·80				
Normal, 1/3080	99·82	86·93	29·42	25·20	70·40	12·89	61·73	12·3
S.E.	2·12	2·20	1·92	0·96				
Septique, 1/3080	100·48	87·40	36·10	24·10	64·38	13·08	63·30	1·7
S.E.	2·22	2·91	1·09	1·43				
Toxin D:								
Welchii D, 1/134	100·33	90·2	58·66	70·5	41·67	10·13	19·70	52·7
S.E.	4·37	3·26	4·18	3·48				
Welchii A, 1/134	100·33	96·4	58·66	80	41·67	3·93	16·4	60·6
S.E.	4·37	4·31	4·18	3·29				

diminution of the inhibitory effect of the toxin on addition of serum. By varying the concentration of the antitoxin added the extent of the diminished inhibition is changed accordingly, as is shown in Table V. Results similar to those reported in Table IV for the toxin of *Cl. welchii*, type A, were obtained with the toxins of the organism, types C and D (Table VI). This table shows that the extent of the reduction of the inhibition due to these toxins is greatest with the antiserum for the type A organism, i.e. it is greatest with that antiserum containing the most α antitoxin. These results support the view that the inhibition of the succinate oxidation produced by the toxins of *Cl. welchii* is due to α toxin.

The effects of heated toxins upon the aerobic oxidation of succinate by small intestine. All the toxins used in this work are heat-labile, except that for the type D organism, the ϵ toxin in which is relatively heat-stable. If the inhibition of the succinate oxidation is due to any of these toxins except ϵ toxin, then it should be removed if the toxins are first heated to 80° for 15 min. The results given in Table VII indicate clearly that the small inhibitions induced by the

Table VII. *Comparison of the effects of toxins, before and after heating for 15 min. at 80°, upon the aerobic oxidation of succinate by small intestine*

The figures, which represent the amount of O_2 absorbed, are the means obtained from experiments replicated five or six times. The standard errors are given in brackets. The same intestinal preparation was used for a particular toxin, heated and unheated. Experimental details in the text.

Toxin	Succinate alone	Toxin added	Heated toxin added	Difference columns 3 and 4
Cl. welchii, type A	100 (3·39)	16 (2·68)	73·2 (3·06)	57·2
Cl. welchii, type C	100 (3·72)	34·6 (1·69)	66·6 (2·16)	32·0
Cl. welchii, type D	99·7 (0·87)	70·2 (2·2)	83·2 (3·01)	13·0
Cl. oedematiens	100·13 (4·85)	73·5 (4·87)	72·83 (4·73)	0·67
Cl. septique	100·13 (2·34)	80·17 (4·44)	78·33 (3·11)	1·84
Cl. tetani	100 (1·35)	94·17 (3·67)	95·60 (2·78)	1·43
Broth ppt. control	99·9 (1·54)	86·44 (2·58)	88·94 (3·20)	2·50

toxins from *Cl. septique*, *Cl. oedematiens* and *Cl. tetani* are unaffected by first heating the toxin but the inhibitions produced by the toxins from the three types of *Cl. welchii* are considerably lessened by pre-heating the toxin. This is further evidence suggesting that the inhibitory action is associated with the α toxin of *Cl. welchii*.

The effect of the addition of the toxin of Cl. welchii, *type A, upon other oxidations by small intestine.* The only substances which appear to be oxidized actively by aqueous suspensions of minced small intestine are succinate, *p*-phenylenediamine and dihydroxyphenylalanine (Table I). If the various toxins are added to mixtures of *p*-phenylenediamine and intestine in the micro-respirometer they all, including the broth precipitate control, inhibit the oxidation nearly 20 % but the toxins of *Cl. welchii*, type A, possesses a slightly more inhibitory action (*vide* Table VIII). The action of this toxin is significantly different from that of the broth precipitate control, but its magnitude is small in comparison with its inhibitory action on the succinate oxidation. Furthermore no inhibition significantly greater than that produced by the broth precipitate is obtained with the toxins of *Cl. welchii*, types C and D.

The addition of the toxin of *Cl. welchii*, type A, to dihydroxyphenylalanine (M/120) and minced small intestine diminishes the rate of oxidation to about 60 % of its normal value, an inhibition greater than that shown with *p*-phenylenediamine but only about half that registered with succinate (cf. Tables IX and II).

Table VIII. *Effects of bacterial toxins upon the aerobic oxidation of p-phenylenediamine in the presence of minced small intestine*

Each experiment was made in quadruplicate. Abbreviations are the same as for Table II, but pp.d. = *p*-phenylenediamine (*M*/54).

Toxin	pp.d.	pp.d. +BP	pp.d. +A	pp.d. +C	pp.d. +D	pp.d. +*Oed.*	pp.d. +*Sept.*	pp.d. +*Tet.*
No. of exps.	10	6	5	5	5	5	5	5
O_2 uptake	100	82·78	72·58	86·37	81·3	79·23	82·15	82·66
S.E.	1·53	1·24	1·85	2·25	2·18	1·52	1·84	1·79
% inhibition	—	17·22	27·42	13·63	18·7	20·37	17·85	17·34
Values for *P* compared with:								
(*a*) Substrate control	—	<0·01	<0·01	<0·01	<0·01	<0·01	<0·01	<0·01
(*b*) Broth ppt. control	—	—	<0·01	0·6–0·7	0·4–0·5	0·5–0·6	0·7–0·8	>0·90

Table IX. *The effect of the toxin of* Cl. welchii, *type A, upon the rate of oxidation of* M/*20 dihydroxyphenylalanine by minced small intestine*

The experimental detail is the same as that described for succinate except that, owing to the slower rate of oxidation, the figures given represent the O_2 absorbed over a period of 80 min. Control experiments with "broth precipitate" gave small inhibitions comparable with that reported for succinate. S.E. = standard error.

	O_2 absorbed	S.E.	No. of exps.	No. of manometers
Substrate alone	100	2·12	5	16
Substrate + toxin	61·4	1·90	5	16

The effect of the toxin of Cl. welchii, *type A, upon the aerobic oxidation of succinate by various tissues.* Minced preparations of small intestine, skeletal muscle, heart muscle, liver, kidney and brain actively oxidize succinate in the Barcroft apparatus. The activity of heart muscle is very great, being 15 times greater than that of skeletal muscle. Despite their varying activities towards succinate the addition of the toxin of *Cl. welchii*, type A, to aqueous suspensions

Table X. *The effect of the addition of the toxin of* Cl. welchii, *type A, upon the aerobic oxidation of succinate by various tissues*

The results given are from single experiments replicated five times and are based on 40 min. readings, except for skeletal and heart muscle (20 min.). The experimental technique is described in the text. All inhibitions except that obtained in one of the brain experiments and that with brain and the broth precipitate control, are significant (i.e. $P = <0{\cdot}01$). Three results are given with brain as they vary considerably from animal to animal. This variation does not occur with other tissues. The tissues were suspended in tap water. The standard errors are given in brackets.

Tissue	Dry wt. of tissue	Q_{O_2}	O_2 uptake: Succinate	O_2 uptake: Succinate + toxin or broth ppt.	% inhibition
(*a*) With toxin:					
Intestine	13·83	11·76	100·11 (0·46)	15·79 (0·35)	84·32
Skeletal muscle	38·00	8·37	100·5 (4·74)	16·5 (2·75)	84
Heart muscle	2·40	126·25	100·25 (3·33)	29·2 (3·54)	71·05
Liver	16·50	11·64	100·33 (3·56)	38·25 (4·77)	61·08
Kidney	7·80	35·00	99·8 (2·85)	25·8 (2·06)	74·00
Brain	7·80	7·20	100·25 (5·12)	109·75 (2·56)	– 9·5
Brain	17·30	13·20	99·83 (3·71)	78·17 (5·41)	21·66
Brain	12·50	12·78	99·60 (2·18)	52·00 (4·04)	47·60
(*b*) With broth ppt.:					
Intestine	14·47	12·06	100 (1·16)	82·41 (1·04)	17·59
Brain	8·0	13·80	99·9 (1·28)	95·75 (2·54)	4·15

of these tissues greatly inhibits the rate of oxidation of succinate with each tissue except brain. The inhibition is generally between 60 and 85%, but with brain the results are indefinite although suggestive that some inhibition, less than that obtained with other tissues, occurs (Table X).

The effects of bacterial toxins upon a dried preparation of tissue enzymes. A dried preparation of "lactic dehydrogenase" was obtained from ox heart as described by Green & Brosteaux [1936]. This preparation was active to some extent with lactate and malate but it was much more active with succinate as its substrate. If the various bacterial toxins are added to mixtures of this enzyme preparation and succinate the rate of oxidation of the succinate is inhibited by the different toxins to an extent comparable with that shown with small intestine (cf. Tables II and XI). The toxins of *Cl. welchii* exert specific inhibitions greater than that

Table XI. *The effect of bacterial toxins upon a dried preparation of succinic dehydrogenase obtained from ox heart*

The abbreviations, experimental technique and method of presentation of results are similar to those described for Table II. A small amount of cytochrome *c*, prepared by the method of Keilin and Hartree [1937], was added to each cup. The number of experiments made was not large.

	S	S+BP	S+A	S+C	S+D	S+*Oed.*	S+*Sept.*	S+*Tet.*
No. of exps.	3	2	3	2	2	2	1	1
No. of manometers	10	6	10	6	6	6	3	3
O_2 absorbed	100·22	82·00	19·64	36·16	57·50	83·00	87·33	75
S.E.	2·93	3·42	1·38	3·87	2·50	2·21	0·33	6·03
% inhibition	—	18·00	80·36	63·84	42·50	17·10	12·67	25·00
Values for P compared with:								
(*a*) Succinate control	—	<0·01	<0·01	<0·01	<0·01	<0·01	0·02–0·05	<0·01
(*b*) Broth ppt. control	—	—	<0·01	<0·01	<0·01	0·8–0·9	0·3–0·4	0·2–0·3

induced by the control broth precipitate. In one or two similar experiments carried out with this enzyme preparation but using lactate or malate as substrate no inhibition was produced by the presence of the toxin of *Cl. welchii*, type A. The rate of O_2 absorption with these substrates was only half that given with succinate and as the experiments have only been done twice their preliminary character must be emphasized.

The effect of the toxin of Cl. welchii, *type A, upon succinic dehydrogenase.* The experiments described in this paper suggest that the α toxin of *Cl. welchii* inhibits the oxidation of succinate by its dehydrogenase. If the activity of the

Table XII. *The effect of the toxin of* Cl. welchii, *type A, upon the anaerobic oxidation of succinate by its dehydrogenase*

The dehydrogenase preparations used were minced small intestine and a dried tissue product prepared from ox heart after Green & Brosteaux [1936]. The usual anaerobic methylene blue technique was followed except that Thunberg tubes with hollow stoppers were employed and the succinate and methylene blue were tipped into the enzyme and toxin mixture after 10 min. anaerobic incubation at 45°. Reduction times (RT) in min. and the relative velocities (V) obtained from the reciprocals of reduction times are shown.

	Small intestine		Dried preparation	
Exp.	RT	V	RT	V
(*a*) Enzyme alone	∞	0	∞	0
(*b*) Enzyme + toxin	36·4	27	∞	0
(*c*) Enzyme + succinate	14·5	69	19	106
(*d*) Enzyme + succinate + toxin	10·4	96	24	83
(*b* + *c*) − *d*	—	0	—	23

enzyme in minced small intestine or in the dried tissue enzyme preparation be examined anaerobically in the presence of the toxin of *Cl. welchii*, type A, no marked inhibition is observed (Table XII). The addition of methylene blue, to act as an "oxygen carrier", to a mixture of small intestine, succinate and toxin in the Barcroft micro-respirometer should reduce the inhibition of the succinate oxidation by the toxin if the latter does not exert its effect upon the dehydrogenase. The results given in Table XIII show that in the presence of 1/3000 methylene blue the inhibition was reduced from 78 to 44%.

Table XIII. *The inhibition of the aerobic oxidation of succinate by small intestine in the presence of the toxin of* Cl. welchii, *type A and methylene blue*

The O_2 absorbed in the presence of methylene blue (1/3000) was slightly more than without it, viz. 110 instead of 100. The experiment was made in quadruplicate. S = succinate, A = toxin of *Cl. welchii*, type A (0·4 mg.), MB = 1/3000 methylene blue and S.E. = standard error.

	S	S+A	S+MB	S+A+MB
O_2 absorbed	100	21·75	100	56
S.E.	1·58	0·48	1·58	2·97
% inhibition	—	78·25	—	44
% reduction of inhibition	—	—	—	43·8

The effect of the toxin of Cl. welchii, *type A, upon the indophenol oxidase of small intestine.* If the presence of indophenol oxidase in small intestine is tested for with Nadi reagent in the way described by Keilin [1929], a very strong positive reaction is obtained. This reaction does not develop in the presence of cyanide but it is practically unaffected by the addition of the toxin of *Cl. welchii*, type A. Similar results were obtained with yeast and with heart muscle suspensions.

Discussion

The results reported in this paper show that those bacterial toxins which contain the α toxin of *Cl. welchii* exert a specific inhibitory action upon the aerobic oxidation of succinate by aqueous suspensions of various minced tissues, e.g. small intestine, skeletal muscle, heart muscle, liver and kidney. A similar effect is experienced with a dried enzyme preparation made from ox heart by the method of Green & Brosteaux [1936]. A few preliminary experiments suggest that the aerobic oxidation of *p*-phenylenediamine by small intestine or of lactate or malate by the dried enzymic product is not inhibited by this toxin. Some inhibition, although only half as much as that with succinate, of the aerobic oxidation of dihydroxyphenylalanine by small intestine is experienced in the presence of the toxin. A number of other toxins of the *Clostridium* group of bacteria do not show any inhibition greater than that induced by the addition of a similarly prepared precipitate from uninoculated broth. The inhibitory action of the toxins appears to be specific to the α toxin of *Cl. welchii* for no toxin without the α toxin produces effects greater than the broth precipitate control, the inhibitory action is destroyed by heating to 80° for 15 min. and it is largely neutralized by the addition of antisera containing antibodies for the α toxin.

In comparison with its toxicity to the live animal the action of the toxin upon this enzyme system of tissues is low, although this may be due in part to our experimental conditions. Working on a basis of the presence of 70% water in a live guinea-pig, the presence of 0·01 mg. toxin of *Cl. welchii*, type A, per g. dry tissue is lethal, whereas for the production of an inhibition of 80% of the activity of the succinate system 1 g. dry tissue (small intestine) requires the

presence of 29 mg. of the toxin. Nevertheless, as an inhibitor of enzymic activity the toxin is potent.

If the inhibitory action of the toxin is examined under anaerobic conditions it is found that no such marked inhibition of the activity of succinic dehydrogenase is depicted. Neither can any inhibitory action of the toxin towards the activity of the indophenol oxidase of small intestine be demonstrated. These results, together with the aerobic oxidation of succinate by small intestine in the presence of toxin and methylene blue suggest that the inhibitory action of the toxin is not exerted directly either upon the dehydrogenase or upon the oxidase but may be associated with an interference to some intermediate link, possibly with an inactivation of some "carrier catalyst".

It has been clearly demonstrated that the α toxin of *Cl. welchii* interferes with a respiratory mechanism associated with the aerobic oxidation of succinate present in various animal tissues. Whether the pathogenicity of *Cl. welchii* is associated in any way with this inhibitory action upon an important respiratory mechanism of tissues is not known, but it is clear from recent progress in our knowledge of the physiological action of certain vitamins, e.g. lactoflavin or ascorbic acid, that the absence of factors associated with respiratory mechanisms of cells can lead to grave disease. Interference with enzymic systems necessary to the supply of energy to tissues might readily lead to subnormality of function, which may open the way to the development of a more active pathological condition. In this way a particular bacterial toxin might facilitate the subsequent invasion of the body by the toxigenic organism itself or by some other organism present at the time.

Summary

1. The toxins obtained from *Cl. welchii*, types A, C and D, inhibit the aerobic oxidation of succinate by aqueous suspensions of minced small intestine.

2. This inhibition is reduced to a considerable extent by the presence of antisera which neutralize the α toxin of *Cl. welchii*.

3. The inhibitory action of the toxins is greatly reduced by heating for 15 min. at 80°.

4. The inhibitory action appears to be due to the α toxin.

5. The toxins of *Cl. oedematiens*, *Cl. septique* and *Cl. tetani* exert no action different from that induced by a precipitate prepared similarly from the broth medium.

6. This inhibitory action is not due to an effect upon either the succinic dehydrogenase itself or the indophenol oxidase, but may be due to an action upon some intermediate carrier catalyst at present unidentified.

7. It is suggested that the pathogenicity of *Cl. welchii* may be associated in part with this inhibitory action of its α toxin upon an important respiratory mechanism of tissues.

8. A brief discussion is given of the importance to invading bacteria of factors which will inhibit enzymic reactions of the host.

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CCXXV. SULPHYDRYL GROUPS AND ENZYMIC OXIDO-REDUCTION[1]

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In an attempt to correlate the observable physical changes that occur within the cell at mitosis with definite chemical processes, the author was led to formulate the working hypothesis [Rapkine, 1931] that cell division is accompanied by configurational changes of the cell proteins resulting in an increase of protein SH groups.

When it was later shown that iodoacetic acid reacts stoichiometrically with SH derivatives like cysteine and reduced glutathione [Dickens, 1933, 1, 2; Rapkine, 1933, 1] as well as with protein SH [Rapkine, 1933, 2] according to the equation

$$R-SH+ICH_2COOH=HI+R-S-CH_2COOH,$$

various attempts were made [Ellis, 1933; Runnström, 1935; Rapkine, 1937] to study the effect of this halogen acid on cell division.

In the meantime results published by various authors not only strengthened the belief that sulphydryl proteins (reduced—partially denatured(?)—proteins) may play in some manner an essential part during mitosis, but also lent more likelihood to the supposition that whenever the activity of a biological system proves to be inhibited by iodoacetic acid it is because this acid acts on SH groups contained in this system. Such are the results obtained on the influence of temperature, pH and concentration on the velocity of interaction of iodoacetic acid and the SH groups of cysteine, glutathione (GSH) and denatured proteins [Rapkine, 1933, 1, 2; 1936]. Also the work of Schubert [1936] has shown that the speed of interaction of iodoacetic acid with tertiary amines is a good deal lower than with SH derivatives. Finally it was possible to show that after having inhibited cell division in yeast cells, by carefully controlled action of iodoacetic acid, these cells could be made to divide again by adding to the culture medium SH compounds [Rapkine, 1937].

It was therefore thought desirable that as many protein systems as possible should be studied *in vitro* with respect to their sensitiveness to iodoacetic acid. Those which should prove to be appreciably inactivated by this acid were to be examined for SH and to see to what extent their activity depended on the integrity of thiol groups within their structure. As prototypes of such systems enzyme preparations appeared to be the most adequate.

The choice of particular enzymes must perforce be an empirical one, since very little is known about those which may be involved in the metabolism of the cell during its division. The choice, however, as to the class of enzymes need not be, as far as our own work on cell division is concerned, empirical. The results obtained in the first paper of this series on the sea-urchin egg [Rapkine, 1931] led us, indeed, to think that the period of predivision is characterized by dehydrogenation processes. Moreover, the lactic acid production in the sea-urchin egg

[1] A preliminary note has already been published [Rapkine, 1938].

was correlated with the presence of SH groups. Our attention was therefore directed to enzymes which catalyse dehydrogenation.

This paper is concerned with the enzyme which catalyses the oxido-reduction between triosephosphate and pyruvate according to the following equation:

$$\text{Triosephosphate} + \text{pyruvate} \rightarrow \text{phosphoglycerate} + \text{lactate}.$$

The presence of coenzyme I is necessary for this oxido-reduction to take place [Meyerhof & Ohlmeyer, 1936; Euler *et al.* 1936]. A detailed study of the nature of the enzyme was made by Green *et al.* [1937] who, in the course of their work, have shown that it is one of a very small number of dehydrogenases that are readily inhibited by fairly low concentrations of iodoacetic acid.

Although there exists already an extensive literature dealing with the thiol groups of hydrolytic enzymes, and how the activity of these enzymes is influenced by oxidation and reduction of their SH groups,[1] the only work of this kind on dehydrogenases has recently been published by Hopkins & Morgan [1938] who brought forward evidence showing that succinic dehydrogenase depends for its activity on the presence of thiol groups.

In the work to be described in the present paper it will be shown that the activity of the enzyme responsible for the oxidation of triosephosphate by pyruvate depends on its state of oxidation-reduction. When oxidized by I_2 or by oxidized glutathione (GSSG) its activity is greatly diminished. When re-reduced by cysteine, reduced glutathione (GSH) or H_2S it is reactivated considerably. Oxidized methylene blue is shown to have no effect whatsoever. With the working hypothesis in mind that it is the SH of the enzyme proper that is being oxidized and reduced, further experiments were made with Cu_2O which is known to react particularly readily with thiol compounds [Hopkins, 1929; Pirie, 1931]. In this case too the evidence obtained shows that the activity of the enzyme can be nearly totally suppressed by Cu_2O and nearly fully regenerated by treatment with H_2S.

However, in order to ascertain that in the experimental conditions to be described the action of Cu_2O, as well as the action of oxidants and reductants, are not concerned rather with the coenzyme than with the enzyme, experiments were run in which the coenzyme was removed by adsorption before any subsequent treatment of the enzyme. The results obtained show that this is not the case; the action of Cu_2O, oxidants and reductants concerns mainly (if not entirely) certain active centres (SH) of the enzyme proper.

I. *Preparation of the enzyme and methods of estimating its activity*

The enzyme is essentially a dialysed aqueous extract of dried acetone powder from rabbit skeletal muscle. It is conveniently prepared by the technique of Meyerhof & Kiessling [1935] according to the directions of Green *et al.* [1937]. The method and the conditions of estimating enzymic activity were essentially the same as those used by the latter authors. Since the oxido-reduction of triosephosphate and pyruvic acid results in the production of acid, the activity of the enzyme which catalyses this reaction may be followed manometrically after addition of $NaHCO_3$ to the mixture containing the enzyme, the substrate and pyruvic acid. Since reduction of pyruvic acid to lactic acid involves no change in acidic groups, the increase in CO_2 pressure is due to phosphoglyceric acid which is formed on oxidation of triosephosphate. There is still however one more condition to be observed if the CO_2 production is to serve as a means of measuring the activity of the enzyme. Hexosediphosphate, which is used throughout these

[1] Full references are to be found in an excellent critical review by Hellerman [1937].

experiments as the source of triosephosphate, is known to give rise to pyruvic acid via phosphoglyceric acid. Fluoride inhibits this reaction, and it must therefore be added in all experiments. Measurements were carried out at 25° and at 30° in the presence or absence of O_2.

II. *Experimental procedure*

(*a*) *Experiments on inactivation of the enzyme by oxidizing with GSSG.* Preliminary experiments have shown that fairly concentrated solutions of GSSG were necessary and the following procedure was adopted: 5 ml. enzyme solution were placed with 2·5 ml. *M*/20 GSSG (final concentration of GSSG is *M*/60) in a Thunberg tube. The tube was evacuated and kept in the water bath at 30° for 3–5 hr. A control experiment was run along identical lines, in which the GSSG was replaced by 2·5 ml. water. The tubes were then opened and 1 or 1·5 ml. of each mixture was pipetted into a Warburg manometer cup. The following reagents were then added: 0·1 ml. *M* pyruvate, 0·3 ml. *M*/2 $NaHCO_3$, 0·7 ml. *M*/10 NaF, and finally 0·4 ml. *M*/10 hexosediphosphate was placed in the side-tube. After temperature equilibration, the hexosediphosphate was tipped from the side-tube into the main compartment of the Warburg cup and the gas evolution was followed.

(*b*) *Experiments on inactivation by oxidizing with* I_2. The action of I_2 is very rapid, and there was no necessity for incubating the enzyme with I_2 for more than 30 min. As the enzyme solution contains reducing substances which react immediately with I_2, it is quite impossible to know what fraction of the I_2 added reacts with the enzyme itself. The procedure adopted consisted in adding I_2 to an aliquot part of the enzyme solution until its reduction became slow. The amount varied with the enzyme preparation, the final I_2 concentration varying according to the experiments from 0·003 to 0·009 *N*. The rest of the procedure was exactly the same as in (*a*) above.

(*c*) *Experiments on inactivation with* I_2 *and GSSG, and reactivation with* H_2S, *GSH and cysteine.* When I_2 was used as the oxidizing agent, the mixture of enzyme and I_2, after keeping in the water bath at 30° for not more than 30 min., was divided into two equal parts, one of which was reserved, whereas the other was treated with H_2S. The H_2S was then eliminated by a current of H_2. As usual a third equal portion treated similarly except for the addition of I_2 served as control. Still another control of fresh enzyme solution was run for the manometric measurements, in order to see the extent of change in activity (if any) the enzyme may have suffered.

When GSSG was the inactivator, the enzyme solution, after incubation with GSSG at 30°, was dialysed for 15 hr. against distilled H_2O at 0° through a cellophane membrane. The enzyme solution was now divided up into three equal lots, *a*, *b* and *c*. To *a*, *M*/10 GSH was added to make the final concentration *M*/35. To *b* an equivalent amount of *M*/10 cysteine and to *c* the necessary amount of water were added. The three lots were now placed in Thunberg tubes; these were evacuated and placed in the water bath at 30° for 3–4 hr. A portion of the mutase solution, which served as a control, was taken through all the stages of treatment, dialysis, incubation, dilution etc., but without added glutathione. Dialysis of the enzyme solution results in a marked loss of activity, and therefore for the manometric measurements another control experiment was run with fresh untreated enzyme solution (adequately diluted) which had been kept in the ice chest at 0°.

One other technical detail deserves mention. Since prolonged dialysis results in a marked loss of activity, a second dialysis after treatment with GSH or

cysteine was not practicable. It is therefore necessary, whenever manometric measurements are done in the presence of O_2, to take into account the absorption of gas due to the oxidation of the excess GSH and cysteine, which in the presence of the enzyme solution is not negligible.

(*d*) *Experiments on inactivation with Cu_2O and reactivation with H_2S.* To 10 ml. enzyme solution 200 mg. Cu_2O were added. The mixture was kept in the water bath at 30° for 15–20 min., with occasional stirring. It was then filtered and divided into two equal parts. One was reserved, while the other was treated with H_2S in the usual manner. As a control a third portion underwent the same treatment (incubation and H_2S) but without addition of Cu_2O. In most experiments however the control portion of enzyme solution was taken through the stage of incubation at 30° only, since experience has shown that treatment with H_2S does not in any way change the activity of the enzyme.

(*e*) *Experiments on inactivation and reactivation of the enzyme after treatment with charcoal to remove coenzyme I.* Merck "Medicinal" charcoal proved to be a good adsorbent of coenzyme I, provided that the charcoal was first washed with distilled water until the wash-water was no longer acid. The enzyme loses most of its activity after two charcoal adsorptions only. On addition of coenzyme I[1] the activity of the enzyme solution is fairly completely restored. Once the enzyme had been charcoal-treated, it was taken through all the stages of inactivation with GSSG, I_2 and Cu_2O. Reactivation experiments were only attempted on charcoal-treated enzyme which had been inactivated by I_2 and Cu_2O. Further experimental details will receive mention in the description of the particular experiments.

III. *Results obtained*

(*a*) *Oxidation and inactivation by GSSG.* Table I shows the results obtained in typical experiments when the enzyme is incubated with GSSG at various concentrations and for various periods of time at 30°.

Table I

After incubation the following amounts of the reagents were pipetted into the Warburg manometer cups for manometric measurements of the enzymic activity; 1·5 ml. treated or untreated enzyme, 0·4 ml. *M*/10 hexosediphosphate, 0·1 ml. *M* pyruvate, 0·3 ml. *M*/2 $NaHCO_3$, 0·7 ml. *M*/10 NaF. The hexosediphosphate was in the side-bulb of the cup and was only tipped into the main compartment after temperature equilibration. The amounts and concentration of the reagents added were, unless otherwise stated, always the same and need not afterwards be repeated.

Final conc. GSSG	Time of incubation hr.	Temp. of manometric measurements		μl. CO_2 in 30 min.
M/100	1½	25°	Enzyme + GSSG	171
			Control without GSSG	256
M/100	1	25°	Enzyme + GSSG	160
			Control	198
M/60	5½	25°	Enzyme + GSSG	11
			Control	157
			Fresh enzyme	146
M/60	5½	25°	Enzyme + GSSG	17
			Control	137
			Fresh enzyme	145
M/60	4½	30°	Enzyme + GSSG	45
			Control	102
			Fresh enzyme	111

[1] I am indebted to Dr T. Cahn and Dr J. Houget for samples of coenzyme I.

As is to be noted, fairly high concentrations of GSSG and rather long periods of incubation are necessary in order to oxidize and hence to inactivate the enzyme. The control which has been put through all the stages of treatment except for the added glutathione, suffers very little loss of activity as can be seen on comparing its activity with that of fresh mutase which had been standing in the ice chest at 0° until the manometric measurements were made.

(*b*) *Oxidation and inactivation by* I_2. I_2 reacts very quickly with the enzyme and the time factor is unimportant. As is seen in Table II it is the I_2 concentration which matters.

Table II

Vol. enzyme ml.	Final conc. I_2	Time of incubation at 30° (min.)	Temp. of manometric measurements		μl. CO_2 in 30 min.
1·2	0·0009 *N*	30	25°	Enzyme + I_2	137
				Control	256
1·3	0·003 *N*	10	25°	Enzyme + I_2	72
				Control	183
1·0	0·009 *N*	30	25°	Enzyme + I_2	40
				Control	198

(*c*) *The action of methylene blue on the enzyme.* It was desirable to know whether some other oxidant than GSSG and I_2 could be made to bring about an inactivation of the enzyme. Oxidized methylene blue was tried. Methylene blue (0·006 *M*) was added to the enzyme solution until there was no more reduction, even after the mixture was evacuated in Thunberg tubes. After 3 hr. incubation in the water bath at 30°, the necessary reagents were added and the activity of the methylene blue-treated enzyme was compared with a control (Table III). No inactivation whatsoever occurred.

Table III

Manometric measurements at 25°.

	μl. CO_2 in 10 min.
Enzyme + methylene blue	106
Control without methylene blue	110

(*d*) *Inactivation with GSSG and reactivation with GSH and cysteine.* As was mentioned in section II, reactivation of GSSG-treated enzyme entailed the elimination of excess GSSG by dialysis which in itself caused a loss of activity of the enzyme. The figures in Table IV illustrate this, as well as the superiority of cysteine over GSH in reactivating the enzyme. This has been met with throughout all the experiments.

Table IV

Incubated with GSSG for 5 hr. Dialysed for 15 hr. at 0°. Incubated with GSH and cysteine for 3½ hr. Manometric measurements at 25°.

	μl. CO_2 in 30 min.
Enzyme oxidized by *M*/60 GSSG, then dialysed	37
Oxidized, dialysed enzyme reduced by *M*/35 cysteine	159
Oxidized, dialysed enzyme reduced by *M*/35 G-SH	81
Control, dialysed	95
Fresh enzyme diluted suitably	127

(*e*) *Inactivation with I_2 and reactivation with H_2S.* As long as I_2 is made to act upon a muscle extract containing both enzyme and coenzyme, the inactivation is very marked, but the reactivation is rather feeble (Table V).

Table V

Incubated at 30° for 10 min. Final concentration of I_2 0·003 *N*. Measurements at 25°.

	μl. CO_2 in 20 min.
Enzyme + I_2	23
I_2-treated enzyme reduced by H_2S	45
Control	88

As will be shown below, the reactivation by H_2S may be nearly complete if the I_2 is made to act upon an enzyme solution from which coenzyme I has been removed by charcoal adsorption.

(*f*) *Inactivation by Cu_2O and reactivation by H_2S.* Although inactivation by Cu_2O is rapid and thorough, the time of contact and the amount of Cu_2O are, up to certain limits, not negligible (Table VI). The degree of reactivation of

Table VI

Enzyme in contact for 3 min. at 30° with Cu_2O before filtration of excess of the oxide. 50 mg. Cu_2O per 5 ml. enzyme solution. H_2S was passed for 5 min.

	μl. CO_2 in 30 min.
Enzyme + Cu_2O	85
Cu_2O treated enzyme decomposed by H_2S	160
Control (treated with H_2S)	257

Enzyme in contact for 20 min. at 30° with Cu_2O before filtration. 100 mg. Cu_2O per 5 ml. enzyme. H_2S passed for 15 min.

	μl. CO_2 in 30 min.
Enzyme + Cu_2O	74
Cu_2O-treated enzyme decomposed by H_2S	243
Control (not treated with H_2S)	268

Cu_2O-treated enzyme by H_2S also varies with time, and it was found that 15–20 min. were quite sufficient for optimum reactivation.

(*g*) *Inactivation and reactivation of enzyme after treatment with charcoal to remove coenzyme I.* The fact that coenzyme I is in some methods of its preparation precipitated as a Cu^+ salt, albeit in acid solution, served as a warning in the interpretation of the results obtained when Cu_2O is made to act upon muscle extracts containing both enzyme and coenzyme I. Is not the inactivation of an enzyme solution by Cu_2O due mainly to its combination with coenzyme I?

It was at once clear that the only way to answer this question, not only for Cu_2O, but also for the other inactivators, was to subject the enzyme to oxidation and reduction after removal of its coenzyme by charcoal adsorption. Preliminary experiments showed to what extent the enzyme suffers loss of activity on adsorption (Table VII). It is certainly not negligible, and the addition of coenzyme I in varying amounts restores, at best, about 80 % of the original activity. As for the residual CO_2 production by the complete system in presence of charcoal-treated enzyme without added coenzyme I, it cannot be due to dismutation of triosephosphate, since in the presence of pyruvic acid dismutation is completely

suppressed [Green *et al.* 1937]. It is very likely due to remaining traces of coenzyme I which have escaped adsorption, and which allow some oxido-reduction between triosephosphate and pyruvate to take place.

Table VII

Manometric measurements at 25°.

	mg. cozymase added		
	1·5	1·0	0·5
	μl. CO_2 in 30 min.		
	(1)	(2)	(3)
Charcoal-treated enzyme + hexosediphosphate + pyruvate + NaF	33	29	55
As above but with coenzyme I	126	130	146
Complete system with untreated enzyme	153	195	178

The results of Table VIII show clearly that under the experimental conditions described the action of Cu_2O cannot be exerted mainly on coenzyme I. If it were through some action on coenzyme I that inactivation of the enzyme by Cu_2O

Table VIII. *Action of Cu_2O on charcoal-treated enzyme*

Charcoal-treated enzyme solution was divided into 7 portions. To portions *a*, *b*, *c* and *d* Cu_2O was added (100 mg. per 5 ml.). After Cu_2O treatment (20 min. at 30°) the excess Cu_2O was filtered off, and whilst *a* and *b* were reserved, *c* and *d* were treated with H_2S. Coenzyme I was now added to *a* and *c*. To portion *e* was added a sample of coenzyme I which had been treated with Cu_2O, and the excess of the latter filtered off. To *f* ordinary coenzyme I was added, whilst no coenzyme was added to *g*. A normal enzyme solution (not charcoal-treated) was now divided into four other portions, *h*, *i*, *j* and *k*. *h* was the normal control; *i*, *j* and *k* were treated with Cu_2O (20 min. at 30°), and after filtration from excess Cu_2O *k* was reserved, whilst coenzyme I was added to *i*. To *j*, instead of coenzyme I, fresh enzyme solution (enzyme + coenzyme) was added. Of each of these various portions of enzyme 1 ml. was pipetted into Warburg manometer cups, and the necessary reagents were added for manometric measurements (see part (*a*) of this section).

	μl. CO_2 in 30 min.
(*a*) Charcoal-treated enzyme + Cu_2O + coenzyme I	8
(*b*) As above but without coenzyme I	19
(*c*) Charcoal-treated enzyme + Cu_2O + H_2S + coenzyme I	102
(*d*) As above without coenzyme I	51
(*e*) Charcoal-treated enzyme + coenzyme I which has been treated with Cu_2O*	99
(*f*) Charcoal-treated enzyme + coenzyme I	170
(*g*) As above without coenzyme I	84
(*h*) Normal (not charcoal-treated) enzyme	183
(*i*) Normal enzyme + Cu_2O + coenzyme I	40
(*j*) Normal enzyme + Cu_2O + fresh enzyme†	197
(*k*) Normal enzyme + Cu_2O	45

* Coenzyme solution was in contact with Cu_2O (20 mg. per ml. for 20 min. at 30°).
† 0·5 ml. of fresh normal enzyme was added.

occurred, then we should expect the charcoal-treated enzyme to be reactivated, even after Cu_2O treatment, by mere addition of coenzyme I to the system. As is shown from Exps. *a* and *b*, this is not the case. Reactivation is only possible when the enzyme has been treated with H_2S (see Exp. *c* and *d*, Table VIII).

That Cu_2O does not react with coenzyme to any appreciable extent is also shown from *e*, where Cu_2O-treated coenzyme I was added to charcoal-treated

enzyme. Further evidence that Cu_2O acts mainly on some active centres of the enzyme proper is shown from Exp. *h*, *i*, *j* and *k*.

Action of I_2 on charcoal-treated enzyme. It was shown in part *e* of this section, that reactivation by H_2S of I_2-treated enzyme is rather small, so long as the I_2 is made to act upon ordinary enzyme solution. As will be seen from Table IX

Table IX

Final concentration of I_2 0·006 *N*. Manometric measurements at 25°.

	μl. CO_2 in 7 min.
Charcoal-treated enzyme + I_2 + coenzyme I	50
As above but without coenzyme I	49
Charcoal-treated enzyme + I_2 + H_2S + coenzyme I	113
Fresh mutase equally diluted	122
As above + I_2	29

good reactivation may be obtained if the I_2 is made to act upon an enzyme solution from which coenzyme I is removed. Moreover, it seems that the inactivation by I_2 of normal enzyme solution is more pronounced than in the case of charcoal-treated enzyme.

Action of GSSG on charcoal-treated enzyme. The results of Table X show the action of GSSG on charcoal-treated enzyme as compared with its action on normal enzyme (untreated with charcoal). The action of GSSG is much more

Table X

The enzyme was incubated with GSSG (final concentration *M*/60) for 4 hr.

	μl. CO_2 in 27 min.
Normal enzyme suitably diluted	228
As above + GSSG	181
Charcoal-treated enzyme + GSSG + coenzyme I	58
As above but without coenzyme I	18
Charcoal-treated enzyme + coenzyme I	147
As above but without coenzyme I	52

pronounced on charcoal-treated enzyme. It must be said that at present no explanation has been found to account for the difference of action of either I_2 or GSSG on normal and charcoal-treated enzyme preparations. Further work is being done in this direction.

Discussion

The strict interpretation of the experiments described is that the enzyme responsible for the catalysis of the oxido-reduction between triosephosphate and pyruvic acid depends for its activity on its own oxidation-reduction state. Acted upon by oxidants, the activity of the enzyme is greatly diminished or nearly suppressed; once inactivated, the enzyme may recover its activity to a great extent by controlled treatment with reductants. In making the tentative hypothesis that these changes in activity of the enzyme are to be ascribed to the oxidation and reduction of the SH groups of the enzyme, one must necessarily cope with the long-standing objections which have already been formulated in the case of hydrolytic enzymes. To begin with, are the thiol groups involved at all? If they are, might there not be, side by side with these, other groups which are at least as essential for the activity of the enzyme? It is, of course, impossible at present to state definitely that there are no other active groups than the

thiol groups in the enzyme studied here. However, the hypothesis that thiol groups are mainly responsible for the activity of the enzyme can be supported by many arguments. Iodoacetic acid inhibits the enzyme readily and thoroughly. Although this acid has been shown to react with other groups, the speed with which it reacts with SH confers on this halogen acid, for practical purposes at any rate, the property of specificity towards thiol groups. The same can virtually be said of Cu_2O, in view of the conditions under which this acts upon the enzyme preparation. The only difference is that an enzyme completely inactivated by Cu_2O can be fully reactivated by H_2S, whilst inactivation by iodoacetic acid is irreversible.

In connexion with the reversible inactivation by GSSG, the arguments brought forward by Hopkins & Morgan [1938] in the case of succinic dehydrogenase are relevant. GSSG is a very mild oxidizing agent, and the fact that the GSSG $\rightleftharpoons$ 2GSH system is very negative in the potential scale makes it very likely that, as an oxidant for proteins, GSSG is specific for the thiol groups of the protein. There is further evidence for this. Hopkins [1925] has shown that in the oxidation of the reduced thermostable residue of tissue by GSSG, the resulting GSH formed is a measure of the pre-existing SH of the proteins. This was later confirmed on reduced heat-coagulated egg-white [Rapkine 1933], where it was, moreover, shown that the amount of pre-existing fixed SH of the protein as measured by the iodoacetic acid technique is identical with that obtained by Hopkins's method. Finally, Mirsky and Anson [1935] proved conclusively that the total number of fixed SH groups in a protein can be approximately measured by the total amount of hydrogen transferred to the oxidant (cystine).

The action of oxidized methylene blue on the enzyme studied here is again in harmony with the thiol group hypothesis. Methylene blue is, like GSSG, a mild oxidizing agent, and although it is high up in the scale of redox potentials it is known to react extremely slowly with thiol groups. This accounts for the ineffectiveness of methylene blue in inactivating the enzyme.

The action of I_2 needs hardly any comment. I_2 is by no means a mild oxidizing agent. Although it oxidizes SH to SS it cannot be said that it reacts readily only with SH groups. The action of I_2 on the enzyme must therefore be considered only in conjunction with the action of other substances such as GSSG, methylene blue and Cu_2O. However, it must be pointed out that inactivation of the enzyme by I_2 is to a large extent reversible by H_2S, especially if the I_2 is made to act on a coenzyme-free (charcoal-treated) preparation of the enzyme.

The behaviour of GSSG towards charcoal-treated enzyme is rather curious. Unless other substances present in the normal enzyme preparations interfere in some manner with the action of GSSG, it is perhaps not unreasonable to think that the coenzyme exerts in some way some protective influence on the enzyme. Once the coenzyme is removed by charcoal, GSSG inactivates the enzyme much more quickly. Whatever the explanation may be, account should be taken of these results whenever similar experiments of oxidation and reduction are carried out on enzymes which require, for their activity, the presence of a coenzyme.

This paper deals with the reversible inhibition of an enzyme which takes part in the first stages of glycolysis. It is therefore not out of place to compare the results obtained on this particular enzyme with those obtained previously by other authors on the effect of inhibitors on glycolysis by whole muscle extracts. Thus Lipmann [1934] showed that Cu in small amounts (10^{-5} to $10^{-4}M$) activates muscle glycolysis, whilst in concentrations of 10^{-4} to $10^{-3}M$ it inhibits

glycolysis. Previously the same author [Lipmann, 1933] had shown that the inhibition of glycolysis by dichlorophenol-indophenol and O_2 could be removed by ascorbic acid and N_2.

Wagner-Jauregg & Rzeppa [1936, 1, 2] obtained evidence that the copper inhibition described by Lipmann [1934] may be reversed by GSH, cysteine, coenzyme I and coenzyme II. More recently a detailed systematic study on the reversible inhibition of muscle glycolysis was made by Gemmill & Hellerman [1937]. Besides metallic compounds the authors also used I_2 as an inactivator of glycolysis, the reactivants used being cysteine, GSH and ascorbic acid. In this same paper [Gemmill & Hellerman, 1937] mention is made of unpublished experiments by Leövey (carried out in 1936) in which the author shows that the conversion of pyruvate into lactate in rabbit muscle pulp is inhibited by I_2 and quinone and restored by H_2S.

The results just enumerated make it tempting indeed to suggest that precisely the enzymic system dealt with in the present paper is one of the systems with which the above-mentioned researches on glycolysis was concerned.

Summary

1. The enzyme which catalyses the oxido-reduction between triosephosphate and pyruvate is inactivated by oxidation with GSSG. If now the excess GSSG is dialysed away, the enzyme can be reactivated by GSH or cysteine, the latter being more rapid in its action.

2. The enzyme is inactivated by I_2. Such an inactivated enzyme can, to a large extent, be reactivated by H_2S. The degree of reactivation is greater if the I_2 is made to act upon an enzyme preparation from which coenzyme has been removed by charcoal adsorption.

3. Methylene blue exerts no influence on the activity of the enzyme.

4. Cuprous oxide completely inactivates the enzyme. Its activity can, however, be fully recovered on regenerating the enzyme by H_2S.

5. It is shown, using charcoal-treated enzyme preparations, that the action of Cu_2O, oxidants and reductants is mainly concerned with active groups of the enzyme itself.

6. The changes in activity of the enzyme under the influence of Cu_2O, oxidants and reductants are ascribed mainly to SH groups. The oxidation or combination of these groups results in an inactivation of the enzyme. The re-reduction or regeneration results in a reactivation of the enzyme. This hypothesis is discussed.

7. The work just reported is discussed in the light of work done by other authors on glycolysing extracts.

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CCXXVI. THE METABOLISM OF ADENINE COMPOUNDS BY *BACT. COLI*

By MARJORY STEPHENSON and ARTHUR REGINALD TRIM

WITH A MICRO-METHOD FOR THE ESTIMATION OF RIBOSE

By ARTHUR REGINALD TRIM

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The importance of nucleic acid derivatives in cell metabolism is becoming increasingly apparent; one may instance the function of muscle adenylic acid in fermentation and its presence as a constituent of coenzymes I and II; the pyrimidine ring occurs in cocarboxylase and uracil has been shown by Richardson [1936] to be essential for the anaerobic growth of *Staph. aureus*. In the metabolism of bacteria it has recently been shown that muscle adenylic acid prevents the loss of activity of *dl*-serine deaminase from a strain of *Bact. coli* [Gale & Stephenson, 1938], whilst adenosine behaves as a coenzyme for aspartase II prepared from the same organism [Gale, 1938].

As a preliminary to studying the mode of action of adenine compounds as coenzymes in the metabolism of bacteria the present study was undertaken in order to elucidate the changes which they themselves undergo in the presence of the enzymes of the organism. Previous studies on various bacteria (including *Bact. coli*) have shown that adenine and guanine are present as nucleotides, nucleosides and free bases in the acid-soluble extracts of the cells and that adenine compounds are more than ten times in excess of guanine compounds. On autolysis of the cells nucleic acid nitrogen decreases and nucleotides, nucleosides and free bases increase [Mesrobeanu, 1936].

It has also been shown that in the case of *Bact. coli* there exist within the bacterial cell enzymes decomposing adenine compounds and that muscle adenylic acid is dephosphorylated and deaminated by various organisms of the Bacteriaceae. Hypoxanthine was identified as the end product of the action of *Bact. coli* on adenine, adenosine, inosine and inosinic acid, and adenyl pyrophosphate was shown to be a cell constituent of the same organism [Lutwak-Mann, 1936]. It now seems probable that the acid-soluble purine derivatives found in the cell originate from the nucleic acid. It has been shown that yeast decomposes its own nucleic acid autolytically and that the adenylic acid thus formed (adenosine-3-phosphoric acid) is rapidly dephosphorylated giving adenosine; the latter can be phosphorylated in its turn by the enzymes of the same organism giving adenosine-5-phosphoric acid ("muscle adenylic acid") and also the di- and tri-phosphates functioning in fermentation [Ostern *et al.* 1937; 1938].

In the present paper the same strains of *Bact. coli* as those employed in previous work were used; three types of decomposition were studied: (1) the dephosphorylation of muscle adenylic acid; (2) the deamination of adenine, adenosine and adenylic acid; (3) the removal of ribose from adenosine and adenylic acid.

Methods

The organisms were grown in flasks of tryptic caseinogen digest broth for 12–14 hr. and centrifuged and washed with water in the usual way. The strength of the bacterial suspension was estimated by the photoelectric turbidimeter [Clifton *et al.* 1935]. The inorganic phosphate was estimated by the method of Fiske & Subbarow [1925], ammonia by the method of Parnas, and ribose by the method described in the appendix. The rates of dephosphorylation, deamination and liberation of ribose are generally expressed as μl. ammonia, phosphate or ribose per mg. dry wt. bacteria per hr. $\left(\mu\text{l.} = \frac{\text{mol. wt. in mg.}}{22{,}400}\right)$. Deamination is also expressed in Q_N (μg. nitrogen/mg. bacteria/hr.). The following stock solutions were used. Phosphate buffer *p*H 7·7 3*M*/450. Adenine 0·602 mg./ml. ($3 \times 33\,\mu$l./ml.). Adenosine 1·15 mg./ml. ($3 \times 33\,\mu$l./ml.). Muscle adenylic acid (adenosine-5-phosphoric acid) neutralized with NaOH 1·54 mg. ml. ($3 \times 33\,\mu$l./ml.). *d*-Ribose 0·670 mg./ml. ($3 \times 33\,\mu$l./ml.). Bacterial suspensions 3–15 mg. dry wt./ml. as convenient.

The decomposition of adenylic acid

Adenylic acid is dephosphorylated and deaminated almost simultaneously, but in all cases so far tried the former process appears to precede the latter.

Exp. 1. 5 ml. each of stock phosphate, adenylic acid (2×stock) and bacterial suspension (3 mg./ml.) were incubated at 37°; 2 ml. and 1 ml. for the estimation of

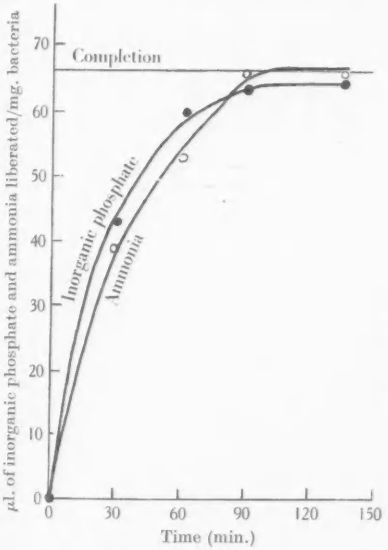

Fig. 1.

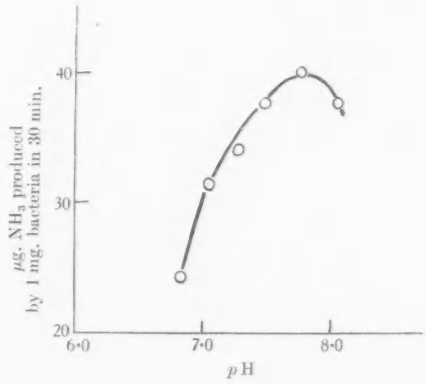

Fig. 2.

Fig. 1. The dephosphorylation and deamination of muscle adenylic acid by *Bact. coli* I.

Fig. 2. Effect of *p*H on the deamination of adenosine by *Bact. coli* I.

ammonia and phosphate respectively were withdrawn at intervals; the results are shown in Fig. 1. The rate of deamination in this experiment corresponds to

$Q_N = 24$. The rate of these reactions varies only slightly with pH increasing by 12 % between pH 6·5 and 8·0 after which it falls off. From the pH curve for the deamination of adenosine (Fig. 2) it appears that the deamination of adenylic acid is controlled by the dephosphorylation and what is actually being measured is the effect of pH on dephosphorylation.

The action of inhibitors

The action of various inhibitors was tested in the hope of separating the dephosphorylation from the deamination. The results are given in Table I.

Table I

Inhibitor	% inhibition: Dephosphorylation	% inhibition: Deamination
1. Phenol 1 %	0	100
2. KCN $M/500$	100	100
3. KCN $M/1000$	100	100
4. NaF $M/50$	60	66
5. Iodoacetate $M/300$	0	0
6. Toluene (saturated sol.)	33	90
7. Chloroform (saturated sol.)	100	100

In the cases of 1, 6 and 7 the bacteria were exposed to the poison for 30 min. at room temperature and then centrifuged and washed before use; 2, 3, 4 and 5 were present during the reaction.

The only useful inhibitor found was 1 % phenol which effects a clean separation of the two mechanisms; the phenol-treated and control organisms are washed twice with distilled water before use.

Exp. 2. 4 ml. each of adenylic acid phosphate and phenol-treated bacteria (6 mg./ml.) were incubated for 30 min. at 37°; 2 ml. were then withdrawn for P and 1 ml. for NH_3 estimations. The remainder was then divided into two 4 ml. samples *A* and *B*. To *A* was added 0·26 ml. fresh bacterial suspension 15 mg./ml. so that the preparation contained 1 mg./ml. fresh bacteria; to *B* 0·26 ml. water was added. After 1 hr. further incubation the phosphate and ammonia estimations were repeated; the usual blank estimations were done (see Table II). Phenol treatment has no effect on dephosphorylation; when rates were measured with phenol-treated and untreated bacteria superimposable curves were obtained.

Table II

Time in min.		NH_2–N μl./2 ml.	Phosphate P μl./2 ml.
30	(Phenol-treated organism)	<8	47·6
90	(Phenol-treated organism)	<8	47·2
90	(Phenol-treated + fresh organism)	46·4	47·2

Complete deamination and dephosphorylation corresponds to 66 μl./2 ml.

The decomposition of adenosine

The adenosine used was obtained from Georg Henning (Berlin) and gave the following figures on analysis:

C 45·11 %, H 4·90 %, N 25·4 % (calculated C 45·10 %, H 4·86 %, N 26·2 %).

Deamination. Adenosine is deaminated more rapidly than adenylic acid, especially by strain I, the difference being less marked in strain II (see Table III).

Table III

	Strain I	Strain II
Average Q_N adenylic acid	20	13
,, adenosine	104	24

Phenol treatment completely inhibits deamination by both strains.

The effect of *p*H is much more marked in the case of adenosine than in that of adenylic acid (see Fig. 2), the optimum being at *p*H 7·75. These facts are consistent with the view that adenosine is the compound actually deaminated.

Removal of ribose

The method used for the estimation of free ribose is described in the appendix. Having there shown that 10 μg. (1·5 μl.) ribose can be accurately estimated after the removal of the bacteria it became possible to study the liberation of this pentose from adenosine.

Exp. 3. 1 ml. each of stock adenosine phosphate buffer and bacterial suspension (3 mg./ml.); 1 ml. of this mixture contains 33 μl. (221 μg.) ribose in the form of adenosine. Control exp. in which adenosine is replaced by water. Incubation at 37°.

1 ml. was withdrawn and deproteinized with 3 ml. 0·2 *N* HCl and 2 ml. of the filtrate used for the estimation of ribose, this is equivalent to 111 μg. ribose which requires 2·00 ml. ceric sulphate (Table IV).

Table IV

	Time in min.	Ceric sulphate ml.
Control	0	0·36
Exp. A	0	0·54
,,	60	0·50
Exp. B	0	0·52
,,	60	0·55

From the results in Table IV it seems that either no ribose is liberated in the experimental period or it is immediately decomposed to substances which do not reduce ferricyanide; Exps. 4 and 5 show that the latter alternative is correct.

Exp. 4. Six test tubes each containing 0·5 ml. stock adenosine, 0·5 ml. water and 3·0 ml. 0·2 *N* HCl were heated in a boiling water bath; at various intervals a tube was removed in which the total free ribose was estimated in the usual way; the results are given in Fig. 3. From this it is seen that 90% of the theoretical amount of ribose is liberated from adenosine and can be estimated after 2 hr. acid hydrolysis. In order to raise the yield of ribose, stronger acid and longer hydrolysis were employed without effect. From the analytical figures for the adenosine used it seems unlikely that this deficit is due to impurity,

Table V

Time in min.	Ribose added μg.	Ribose estimated μg.	Furfuraldehyde test
0	100	99	0
30	100	93	0
60	100	97	(+)
120	100	104	(+)
180	100	98	(+)

though a control in which 100 μg. ribose were heated under the same conditions shows a higher recovery of ribose and a slight furfuraldehyde reaction by the aniline acetate test (Table V).

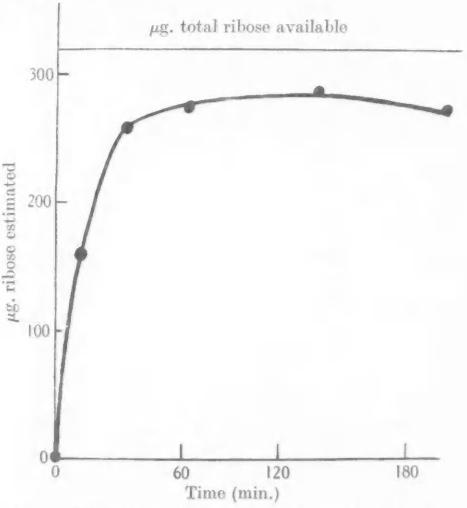

Fig. 3. Hydrolysis of adenosine by 0·2 *N* HCl at 100°.

We next tried to determine whether the ribose is actually split off from the adenosine as a result of the bacterial action.

Exp. 5. Equal vols. of stock adenosine, phosphate buffer (3*M*/450 at *p*H 7·7) and bacterial suspension 3 mg./ml. were incubated at 37° (1) aerobically, (2) anaerobically; 2 ml. samples were removed at intervals and 6 ml. 0·2 *N* HCl added, shaken with kieselguhr and filtered; the ribose was estimated in the filtrate after 2 hr. acid hydrolysis (*A*); 2 ml. of the filtrate without acid hydrolysis were used as control (*B*). $A-B$ $(=C)$ is the ribose present as adenosine.

If C_0, C_{15} and C_{30} etc. are the quantities of ribose found after the action of the bacteria for 0, 15 and 30 min. C_0–C_{15} is the amount of ribose split off from adenosine in 15 min. as the result of bacterial action.

Table VI

Time min.	μg. ribose removed/mg. bacteria	
	Aerobically	Anaerobically
0	0	0
15	42	45
30	76	76
60	182	177

Total available ribose = 182 μg. (90 % of theoretical, see Exp. 4).

Table VI shows that in 60 min. 100 % of the ribose of adenosine has disappeared yet no ribose can be detected in the culture fluid at any period as shown in Exp. 3. This implies that ribose must be destroyed as quickly as it is liberated, so a comparison was made of the rate of destruction of free ribose and that of ribose combined in adenosine.

Exp. 6. 2 ml. stock buffer and 2 ml. bacterial suspension (3 mg./ml.) were incubated (*a*) with 2 ml. stock adenosine, (*b*) 2 ml. stock ribose.

2 ml. samples from (*a*) and (*b*) were removed at intervals and the ribose in each estimated in (*a*) after acid hydrolysis (as in Exp. 5) in (*b*) direct. From these results the rate of decomposition of ribose (*a*) as adenosine, (*b*) as free ribose were determined both for organisms grown on broth and on glucose broth. The results appear in Table VII.

Table VII

Rate of fermentation of ribose μg./mg. bacteria/hr.	Free	As adenosine
Organism grown on plain broth	15	198
,, ,, glucose ,,	20	204

Both inhibited 100% by treatment of organism with 1% phenol.

The rate of disappearance of ribose in combination as adenosine being so much greater than when free, confirmation of the phenomenon was sought by the manometric measurement of the fermentation. The course of the fermentation was followed by measuring the total gas evolved in bicarbonate buffer, which may be CO_2 or H_2, and also gas liberated by fermentation acid. The experiments were done in Warburg manometers.

Exp. 7. Each manometer contained 3 ml. total liquid.

0·2 ml. *M*/50 substrate (side bulb),
1 ml. bicarbonate buffer *M*/13,
1 ml. bacterial suspension (3 mg./ml.),
0·8 ml. water.

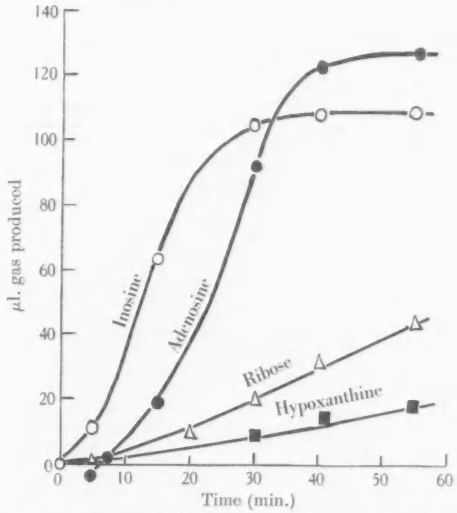

Fig. 4. Total gas produced in bicarbonate buffer from adenosine, inosine and free ribose (*Bact. coli* I)

The usual controls were performed. Gas production from the following substrates was measured: adenosine, inosine, *d*-ribose and hypoxanthine; the results are given in Fig. 4. From this it is seen that the rate of fermentation of free

ribose is about 0·1 that of ribose combined in adenosine and in inosine. (The rate of fermentation of adenosine is the same in the presence and absence of phosphate.) The results of Exp. 6 are therefore corroborated and the disappearances of ribose in the free state and in adenosine are seen to be due to fermentation.

The deamination of adenine

Adenine is very slowly deaminated (Q_N 3–5); this rate is increased two- to six-fold by the presence of adenosine or inosine.

Exp. 8. Solutions were made up as in Table VIII; 2 ml. from each solution were removed at intervals and the NH_3-N estimated; the results are given in Table IX.

Table VIII

	ml.	ml.	ml.	ml.	ml.
Bacterial susp. 3 mg./ml.	3	3	2	3	2
Adenine stock	3	3	0	3	0
Adenosine stock	0	0·3	0·2	0·6	0·4
Water	1·5	1·2	2·8	0·9	2·6
Buffer 3 *M*/225	1·5	1·5	1·0	1·5	1·0
Total vol. ml.	9	9	6	9	6
No. of exp.	1	2	2*a*	3	3*a*

Table IX. NH_3-N

Exp. no. ...	1		2		2*a*		2–2*a*		3		3*a*		3–3*a*	
Time min.	mg.	µl.	mg.	µl.	mg.	µl.	mg.	µl.	mg.	µl.	mg.	µl.	mg.	µl.
0	<0·005	<8	<0·005	<8	<0·005	<8	0	0	0·005	8	<0·005	<8	0	0
30	<0·005	<8	0·0279	44·5	0·005	8	0·0274	36·5	0·035	56	0·010	16	0·025	40
90	0·007	11	0·036	55	0·005	8	0·031	47	0·0457	72	0·040	16	0·0357	56

Table X

	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Bacterial susp. 3 mg./ml.	1	1	1	1	1	1	1
Adenine stock sol.	1	1	1	1	1	1	1
Adenosine $\frac{\text{stock}}{10}$ sol.	0	0·5	0·25	0·05	0	0	0
Inosine $\frac{\text{stock}}{10}$ sol.	0	0	0	0	0·5	0·25	0·05
Water	0·5	0	0·25	0·45	0	0·25	0·45
Buffer 3 *M*/225	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Total vol.	3	3	3	3	3	3	3
NH_3-N in 35 min.							
mg./2 ml.	0·005	0·0178	0·0141	0·0124	0·0176	0·0139	0·0114
µl./2 ml.	8	28·5	22·6	19·8	28·2	22·2	18·3

Table XI

Concentration = x (33 µl./ml.)

x Adenine	x Adenosine	x Inosine	Q_N Adenine
1	0	0	4·2
1	0·005	0	10·6
1	0	0·005	9·7
1	0·025	0	12·0
1	0	0·025	12·0
1	0·05	0	15·0
1	0	0·05	15·0
1	0·1	0	25·0
1	0·2	0	27·0

Effect of adenosine and inosine on the rate of deamination of adenine by Bact. coli *I*. Adenylic acid also has a catalytic effect on the deamination of adenine but slightly smaller than that of adenosine (Fig. 5); whilst the effect of ribose is comparatively insignificant, a concentration equivalent to that of adenine merely doubling the rate of deamination.

The effects of lower concentrations of adenosine and of inosine on the rate of deamination of adenine were then tested; the results are given in Table X, and the combined results from Tables IX and X shown in Table XI.

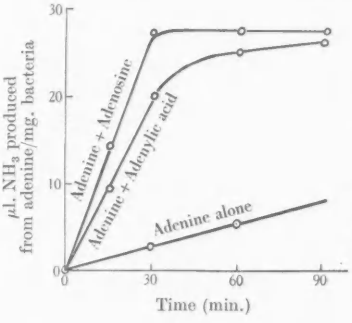

Fig. 5.

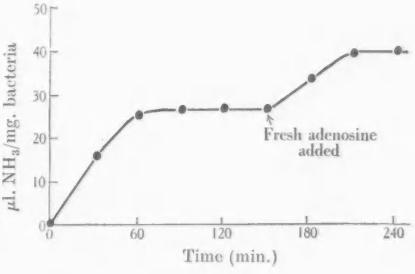

Fig. 6.

Fig. 5. The effect of adenosine and adenylic acid (3·3 μl./ml.) on the deamination of adenine (33 μl./ml.).

Fig. 6. Effect of adenosine (3·3 μl./ml.) on deamination of adenine (99 μl./ml.).

The catalytic effect of adenosine falls off with time, owing probably to its decomposition by the liberation of ribose; if fresh adenosine is added the initial rate is again restored (Fig. 6). The decreasing catalytic effect due to concentration shown in Table XI is partly accounted for in this way.

It is premature to discuss the nature of the catalytic effect of adenosine on the deamination of adenine. It is conceivable that the NH_2 group of adenine may be transferred to inosine which is then rapidly deaminated. The maximum rate of deamination of adenine in presence of adenosine is still less than that of adenosine, so evidence thus afforded is not inconsistent with this view. On the other hand Gale [1938] has recently found the rate of deamination of aspartic acid by aspartase II from the same organism increased by the addition of adenosine far beyond the rate of deamination of adenosine alone; in this case therefore the above hypothesis cannot hold and further work is necessary to arrive at the mechanism involved.

Lutwak-Mann [1936] showed that hypoxanthine was formed as end product of the action of *Bact. coli* on adenine, adenosine and adenylic acid. We have confirmed this.

Isolation of hypoxanthine from the deamination of adenine

The hypoxanthine was isolated as a silver nitrate compound [Bruhns, 1890]; 200 mg. adenosine, 100 ml. *M*/50 phosphate at *p*H 7·7 and 5 ml. bacterial suspension containing 100 mg. bacteria were incubated aerobically at 37°; after 3 hr. 2 ml. adenosine solution (66 μl./ml.) were added; the course of the reaction

was followed by NH_3 estimations; fresh adenosine was added at intervals of $1\frac{1}{2}$ hr. till 700 μl. had been added. At the end of 24 hr. deamination had stopped having reached 70% completion. 2 ml. 10*N* HCl was added, the whole shaken with kieselguhr and filtered; the clear filtrate was evaporated *in vacuo* to about 30 ml. and treated with excess of sodium picrate to remove the unchanged adenine, the adenine picrate was filtered on a Büchner funnel and washed. The filtrate and washings were then treated with excess ammonia and completely precipitated with silver nitrate. The precipitate (consisting of silver chloride, phosphate and a silver hypoxanthine derivative) was filtered on a Büchner funnel, washed with hot water and the residue boiled with 50 ml. HNO_3 sp. gr. 1·1; the acid was decanted off and the precipitate filtered whilst hot and re-extracted several times with HNO_3. 0·25 g. $AgNO_3$ in HNO_3 was added to the filtrate which was left 24 hr. The precipitate was filtered off and dried at 100° for 30 min. and again taken up and reprecipitated with $AgNO_3$ and dried. The analysis was as follows:

	Found %	Calculated %
C	20·52	19·68
H	1·65	1·31
N	22·8	22·95
Ag	34·78	35·49

Hypoxanthine can also be detected and estimated by the use of xanthine oxidase.

Exp. 9. 3 ml. adenylic acid stock ×2. 3 ml. phosphate 3*M*/450 *p*H 7·7. 3 ml. bacterial suspension 3 mg./ml. 2 ml. samples were taken at intervals and 1 ml. *N* HCl added and the whole shaken with kieselguhr and filtered. 1·5 ml. filtrate, 0·5 ml. *N* NaOH and 1 ml. *M*/5 phosphate *p*H 7·0 were put in a Warburg manometer vessel and 0·5 ml. of a 20% neutralized xanthine oxidase solution [Dixon & Kodama, 1926] in the side bulb. 1 μg. hypoxanthine in presence of the xanthine oxidase preparation took up 0·24 μl. O_2. With all substrates tested

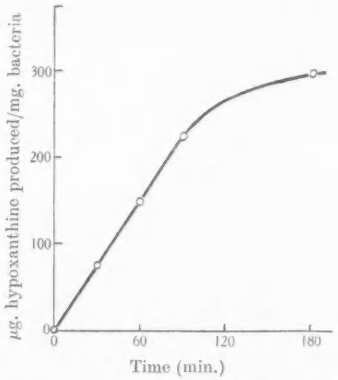

Fig. 7. Production of hypoxanthine from adenylic acid by *Bact. coli* I.

(adenylic acid, adenine and adenosine) about 70% appeared as hypoxanthine (see Fig. 7). The bacterial suspension had a negligible xanthine oxidase activity ($Q_O = 1·5$).

APPENDIX

A MICRO-METHOD FOR THE ESTIMATION OF RIBOSE IN THE PRESENCE OF BACTERIAL SUSPENSIONS

By A. R. TRIM

Ribose may be estimated by a modification of the ceric sulphate titration method for reducing sugars previously worked out for blood sugar estimation by Giragossintz *et al.* [1936]. By this method the sugar is allowed to reduce ferricyanide to ferrocyanide at an alkaline reaction and then the ferrocyanide is re-oxidized with standard ceric sulphate after making strongly acid with concentrated H_2SO_4. Xylene cyanol FF is used as an indicator. The sugar does not reduce its equivalent of ferricyanide according to the stoichiometric relation represented by the equation

$$R.CHO + 2\ Fe\ (CN)_6''' \rightarrow R.COOH + 2\ Fe\ (CN)_6'''',$$

but the extent of reduction of the ferricyanide varies somewhat with the conditions of the experiment; this being the case the estimations must be made under standard conditions and a calibration curve constructed. The advantage of this method is that only the ceric sulphate need be accurately standardized; once standardized the stock solution may be kept for months without deteriorating. Provided that they are in excess, the other reagents need not be used in precise quantities, and changes during storage, so important in certain other methods, are without effect.

Before proceeding with the estimation it is necessary to remove the bacteria; trichloroacetic acid cannot be used for this purpose as during the subsequent procedure it produces substances which reduce ferricyanide. HCl is free from this disadvantage and in concentrations of 0·2 *N* to 2·0 *N* followed by filtration through kieselguhr gives a clear filtrate which on treatment with trichloroacetic gives no further precipitate.

The ribose used was supplied by Dr Georg Henning of Berlin and gave the following constants:

M.P. 85–87°; rotation $[\alpha]_D^{18°} = 219$, kindly determined by Dr D. J. Bell. The analysis by Dr G. Weiler gave the following results:

C%	H%	
39·87	6·67	Found
40·0	6·58	Calculated

Ash none.

PROCEDURE

1 ml. bacterial suspension containing 10–250 μg. ribose was taken and 3 ml. 0·2 *N* HCl added, shaken with kieselguhr and filtered on a pleated filter paper; 2 ml. of filtrate, 2 ml. of 0·8 % potassium ferricyanide and 2 ml. of 15 % sodium carbonate were heated on a boiling water bath for 5–10 min., cooled thoroughly and one-third to one-half the total vol. 50 % H_2SO_4 added; the end point of the final titration requires this large excess of acid. To the cold acid solution a few drops of 0·2 % xylene cyanol FF were added and the solution titrated against standard ceric sulphate in *N* H_2SO_4; in these experiments 0·002 *N* ceric sulphate was used.

It was found that the reduction of the ceric sulphate for any one batch of bacteria was constant and remained unchanged when the suspension was

incubated. It was therefore possible to correct for the reduction due to the reagents and bacteria by running blank controls for each set of experiments.

A curve showing the relation between μg. ribose and ml. standard ceric sulphate was constructed over the range 0–250 μg. ribose at intervals of 50 μg. using 0·002 N ceric sulphate. The curve showed a linear relation in which 1 μg. ribose was equivalent to 0·018 ml. ceric sulphate. The concentration of the ceric sulphate is quite arbitrary, so that the amount of ribose is given by the equation:

$$x = \frac{V \times N}{0{\cdot}18 \times 0{\cdot}002}.$$

Where x = μg. ribose, N = normality of the ceric sulphate and V = ml. ceric sulphate used. Similar results were obtained over the range 0–100 μg. ribose at 10 μg. intervals. Table XII shows a typical set of results:

Table XII

Vol. of solution ml.	Ribose μg.	ml. ceric sulphate		ml. ceric sulphate equivalent to ribose		ml. ceric sulphate / μg. ribose
		A	B	A	B	
2	0	0·47	—	—	—	—
2	0	0·47	—	—	—	—
2	0	—	0·62	—	—	—
2	0	—	0·63	—	—	—
2	50	1·31	1·46	0·86	0·84	0·0172
2	100	2·21	2·36	1·75	1·74	0·0175
2	150	3·08	3·23	2·61	2·61	0·0174
2	200	4·12	4·27	3·65	3·65	0·0183
2	250	4·90	5·05	4·43	4·43	0·0177

In both A and B bacteria were originally present and were removed before the estimations were made. In A the original solution contained 1 mg. of bacteria per ml. and in B 2 mg. per ml.

Summary

1. Muscle adenylic acid is deaminated and dephosphorylated by *Bact. coli* (two strains). Dephosphorylation appears to precede deamination.

2. Deamination is completely inhibited by treatment of the washed suspension of *Bact. coli* with 1% phenol; this treatment has no effect on dephosphorylation.

3. Deamination of adenosine is faster than that of adenylic acid (Q_N about 100 and 20 respectively with strain I) that of adenine is the slowest of the three (Q_N about 5 using strain I).

4. Adenosine increases the rate of deamination of adenine 6–7 times.

5. Adenosine (and inosine) lose ribose when acted on by *Bact. coli*. This ribose can be shown to disappear from its combination in adenosine but cannot be detected in the free state. This disappearance is shown to be due to fermentation.

6. The rate of fermentation of ribose in adenosine (or inosine) is about 10 times that in the free state.

7. The activity of strain II was less than that of strain I but the order of the activities was the same.

8. A micro-method for the estimation of ribose is appended.

Our thanks are due to Prof. Sir Frederick Hopkins for his interest in our work, and to Mr S. W. Cole for advice on a number of points in connexion with the estimation of ribose.

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CCXXVII. ENZYME FORMATION AND POLYSACCHARIDE SYNTHESIS BY BACTERIA

III. POLYSACCHARIDES PRODUCED BY "NITROGEN-FIXING" ORGANISMS

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In a previous paper [Cooper & Preston, 1937] attention has been drawn to the question of polysaccharide formation by the nitrogen-fixing organisms, *Rhizobium radicicolum*, the root-nodule bacterium associated with the Leguminosae, and the soil organism, *Azotobacter chroococcum*. In the present paper we have confirmed the previous observations and have obtained preliminary data on the purification and properties of these interesting polysaccharides. In themselves these products are of considerable importance since they probably constitute a certain proportion of the so-called "humic substances" in soil. From our point of view they are typical examples of nitrogen-free bacterial mucilages. As already indicated, a study of the chemical and physical properties of the *Rhizobium* polysaccharides and the recognition of glucose and uronic acids among their cleavage products places them in the same class of compound as the specific polysaccharides of types II and III *Pneumococcus*. We have now shown that the *Azotobacter* polysaccharide also belongs to the same class. As a result of further study of the metabolism of these organisms, the polysaccharides are now reasonably accessible and in such quantities as to enable their chemical investigations along classical lines to be undertaken. Later it is hoped to apply the information gained to research on important questions of immunological specificity.

In this study, careful attention has been given also to the urgent problem of the possible contamination of specific polysaccharides by extraneous carbohydrate material from the media on which the organisms are cultivated. It is generally known that organisms grown on an agar medium are liable to extensive contamination with agar-polysaccharides. This danger has been particularly emphasized by Morgan [1936] in the case of the specific polysaccharide of *B. dysenteriae*. In their studies on a mucoid polysaccharide from *Streptococcus*, Heidelberger *et al.* [1937] were careful to show that no polysaccharides from the sterile broth medium were precipitated under the conditions used for isolating the bacterial polysaccharide.

In order to minimize the danger of contamination due to mechanical dislodging of agar particles we raised the agar concentration in the medium to 1·7%. In Roux bottles this gave a comparatively hard surface at 37°, and after incubation for several days the washings from these bottles were shown to contain a negligible amount of agar. Agar is constituted mainly of galactose residues so that we were particularly fortunate in being able to show that the poly-

saccharides from the nitrogen-fixing organisms were free from galactose. Of greater importance, however, was the observation that the organisms formed the polysaccharide characteristic of each on either agar or gelatin media; so it is considered that our purified products are free from extraneous carbohydrates.

Two other nitrogen-fixing organisms, *B. urea* (Beijerinck) and *B. trauffauttii*, were investigated in regard to polysaccharide formation. They grew vigorously on an agar medium but when washed off and precipitated no polysaccharides could be isolated.

Rh. radicicolum *polysaccharide*

The organism was an active culture of the clover strain kindly supplied by Dr H. Nichol of Rothamsted Experimental Station.

Extensive investigations on the growth of the organism in liquid media did not lead to a satisfactory method of polysaccharide production. The viscous material obtained was very difficult to purify and the yields were poor. On solid media, however, growth was vigorous and resulted in prolific polysaccharide formation which, moreover, was continuous over a long period. The striking advantage in the production of this type of polysaccharide on solid media may have an important application in large scale preparation of those pathogenic organisms whose antigenic properties may depend on the elaboration of complex polysaccharides.

After numerous investigations of sources of available nitrogen and carbon and of possible growth stimulators, a reliable agar medium for producing the polysaccharide was established. On a gelatin medium the same polysaccharide was produced but the growth was slower and the yield inferior.

Crude *Rh. radicicolum* polysaccharide was grossly contaminated with water-insoluble material, tenaciously held in colloidal suspension. Much of this impurity consisted of bacterial protein the greater part of which was removed by repeated filtration of a dilute aqueous solution through a pad of kieselguhr. Final traces were eliminated by exhaustive alcoholic fractionation. A typical sample of the polysaccharide was obtained as a white fibrous mass $[\alpha]_D - 17°$ in water; ash, 2%; uronic anhydride, 18%. Its hydrolysis with N H_2SO_4 was complete in about 8 hr. at 100°. From the hydrolysate ($[\alpha]_D + 18°$) crystalline *d*-glucose (67% yield) and the barium salt of a uronic acid (23% yield) were isolated. The latter had $[\alpha]_D + 11°$, Ba 16·0%, and was probably the salt of an aldobionic acid. Further investigations of this acid and the polysaccharide will be reported later.

The Azotobacter *polysaccharide*

The organism used in this study was a culture of *Azotobacter chroococcum* freshly isolated from soil and which gave a vigorous production of polysaccharide on gelatin or agar slopes. Initial investigation of its metabolism was directed towards the finding of a suitable liquid medium in which to produce the polysaccharide. Yields of purified material were so small, however, that this method was abandoned. When grown on solid gelatin or agar media results were much more consistent and the purified polysaccharides from each medium were identical. The agar medium was adopted for large scale work. This *Azotobacter* polysaccharide was purified by the method described for the *Rh. radicicolum* polysaccharide, which it resembled very closely in physical properties. Its content of bacterial protein was comparatively small and its purification was readily effected. A purified dried sample was a white asbestos-like mass, which dissolved in water forming a viscous clear solution $[\alpha]_D - 2°$; ash, 8%; uronic anhydride 4%. Hydrolysis of the polysaccharide was effected with $N/2$ H_2SO_4

at 100° in about 10 hr. From the hydrolysate ($[\alpha]_D + 26°$), crystalline *d*-glucose in 87 % yield and the barium salt of a uronic acid in 3 % yield were isolated. The recognition of a small percentage of a uronic acid as a constituent of the *Azotobacter* polysaccharide molecule places this polysaccharide in the same class as the type II and III *Pneumococcus* polysaccharides. Further investigations of molecular structure will be reported later.

Experimental

I. Rhizobium radicicolum *polysaccharide*

The organism was supplied by Dr H. Nichol (*vide supra*). It remained quite active as a gum producer on suitable medium over a long period of subculturing. The basal culture medium was essentially that previously employed [Cooper & Preston, 1937] and had the following composition:

Lucerne-root extract agar (dissolved in 500 ml. of tap water): K_2HPO_4 0·5 g., $MgSO_4$, $7H_2O$ 0·20 g., NaCl 0·20 g., $MnCl_2$ 0·01 g., $FeCl_3$ 0·01 g., $CaCO_3$ 3·0 g., sucrose 20 g., asparagine 5 g.

Powdered lucerne root (5 g.) was boiled with distilled water (500 ml.) for 1 hr., allowed to stand overnight and filtered through cotton wool. This extract was added to 500 ml. of hot basal medium in which was dissolved powdered agar (18 g.). Transference of the medium in 20 ml. portions to boiling tubes was immediately carried out. Stock cultures of the organism were maintained on 5 ml. slopes.

Investigations on liquid media. Using the above basal salt medium, additional substances in varying amounts were added in an endeavour to study the factors influencing the production of polysaccharide. In general the medium was distributed in small conical flasks, inoculated from slopes and incubated for about 10 days. The mucilaginous growth was diluted with distilled water, filtered through cotton wool and the solution run into twice its volume of alcohol. The polysaccharide which usually rose to the surface, was isolated and examined. Production of polysaccharide from media containing varying concentrations of sucrose was first studied. Observations showed that a 5 % sucrose concentration gave maximum yields. Other constituents were now varied and the following conclusions were reached:

(*a*) Addition of a maximum amount of 1 % beet molasses was sufficient to produce growth and polysaccharide formation, the latter reaching a maximum in 10 days at 30°. If molasses was employed, the medium developed a very dark colour during sterilization and the polysaccharide formed was difficult to separate from pigmented material. Initial clarification of the molasses could readily be effected by charcoal but this treatment reduced considerably its power as a growth stimulator.

(*b*) Demerara and beet sugars, alcoholic extracts of peptone or molasses were active in producing growth, whilst malt extract, maple syrup and lecithin were without effect.

(*c*) Addition of 0·02 % of caffeine stimulated polysaccharide formation but it could not replace asparagine as a nitrogen source.

For larger scale work the following medium was employed: KH_2PO_4 2 g./l., $MgSO_4$, $7H_2O$ 0·2 g./l., NaCl 0·2 g./l., $MnCl_2$ 0·01 g./l., $FeCl_3$ 0·01 g./l., $CaCO_3$ 5·0 g./l., asparagine 1·0 g./l., caffeine 0·2 g./l., sucrose 50 g./., alcoholic extract of molasses 10 ml./l.

200 ml. amounts of this medium, distributed in 500 ml. conical flasks were steamed on 3 successive days and inoculated with 1 ml. of an active 48 hr. culture

of *Rh. radicicolum* (clover variety) growing in the same medium. The flasks were incubated at 30° for 10 days. The crude polysaccharide was precipitated by pouring the viscid solution into excess of ethyl alcohol (2 vol.) containing a few drops of calcium chloride solution. The gummy fibrous mass which rose to the surface was isolated, washed with absolute alcohol and ether and dried in a vacuum. It formed a stable white asbestos-like mass. Maximum yield of crude material, 300 mg./l. of medium.

Products made on liquid medium were grossly contaminated with protein material and ash (mainly phosphate). After acid hydrolysis the polysaccharides were shown to be of variable composition and the method was abandoned.

Production of the polysaccharide on a solid gelatin medium. A medium having the following composition was prepared: KH_2PO_4 1·0 g./l., $KHPO_4$ 1·0 g./l., NaCl 0·2 g./l., $MgSO_4$, $7H_2O$ 0·2 g./l., $CaCO_3$ 5·0 g./l., $Fe_2(SO_4)_3$ 0·01 g./l., $MnCl_2$ 0·01 g./l., $CaSO_4$ 0·01 g./l., asparagine 1·0 g./l., sucrose 40·0 g./l., gelatin 150·0 g./l.

It was dispersed in 200 ml. amounts in Roux bottles, steamed on three successive days and inoculated by means of small sterile pipettes with a 48 hr. culture of the organism. After incubation at 20° for 14 days the polysaccharide was washed off with distilled water, the solution filtered through kieselguhr and purified by the method described later. Yield of crude material 50 mg./Roux bottle. It readily dissolved in water forming a viscous clear solution, $[\alpha]_D - 20°$ (*c*, 0·21). Ash, 2·0%. On hydrolysis with $N/2$ H_2SO_4 at 100° the following changes were observed: initial value $[\alpha]_D - 20°$, 45 min. $+5·0°$, 70 min. $+10°$, 120 min. $+12°$, 220 min. $+15°$ (equilibrium value).

Production of the polysaccharide on agar medium. Preliminary experiments were carried out on 20 ml. slopes in boiling tubes. After a suitable incubation period the mucilaginous growth was diluted with water, filtered through cotton wool and dropped into two volumes of alcohol. The precipitated polysaccharide was isolated in the usual manner and purified by alcoholic fractionation. It was identical with the polysaccharide grown on gelatin medium. $[\alpha]_D - 18°$ (*c*, 0·5); ash 5·0%. On hydrolysis with $N/2$ H_2SO_4 at 100° the following changes were observed: initial value $[\alpha]_D - 18°$, 100 min. $+8°$, 170 min. $+15°$, 220 min. $+18°$ (equilibrium value). No mucic acid could be obtained after nitric acid oxidation of the polysaccharide.

Control experiments were carried out: (*a*) with Roux bottles of identical agar medium but uninoculated; (*b*) using similar Roux bottles inoculated with two nitrogen-fixing organisms, *B. ureae* (Beijerinck) and *B. trauffauttii*. These bottles were incubated and treated in a manner similar to that used for isolating the *Rh. radicicolum* polysaccharide. Although the two organisms grew well on the medium in no case was any polysaccharide obtained.

From these experiments it was apparent that contamination by agar was slight, and after a series of experiments to determine optimal conditions whereby the yield per Roux bottle was increased to 0·25 g. it was considered advantageous to use the following medium for large-scale production: (dissolved in 500 ml. of water) asparagine 1·0 g., K_2HPO_4 1·0 g., $MgSO_4$, $7H_2O$ 0·2 g., NaCl 0·2 g., $MnCl_2$ 0·01 g., $FeCl_3$ 0·01 g., sucrose 40·0 g., caffeine 0·2 g., agar 17·0 g., lucerne root extract 500 ml.

The organism was grown on this medium distributed in 200 ml. amounts in Roux bottles and incubated at 25° for 10 days. The heavy growth was removed by addition of a few ml. of distilled water and rubbing gently with a glass rod. The resultant viscous solution was filtered through glass wool, and the polysaccharide isolated as usual by alcoholic precipitation.

Purification and general properties of the polysaccharide. The material, resuspended in water, formed a viscous opalescent colloidal solution which contained appreciable amounts (60 %) of bacterial "debris" in suspension. This "debris" was mainly of a protein nature and it could not be removed by the usual centrifugal methods. Attempts to clear the solution by digestion with trypsin, pepsin or papain were partially successful, but this method was abandoned on realization that commercial preparations of these enzymes contained variable but usually significant amounts of carbohydrate material.

Intensive drying of the crude polysaccharide followed by fractional alcoholic precipitation from aqueous solution removed the bulk of suspended material which was separated in the fractions isolated at low alcohol concentrations. This method was tedious and the following "kieselguhr filtration method" was more convenient and of general application. A small amount of kieselguhr was suspended in distilled water and flooded on to a large Büchner funnel. More kieselguhr was stirred into a very dilute solution of the polysaccharide which was filtered several times under slight vacuum through the prepared filter. The filtrate was evaporated *in vacuo* to a syrup, poured into excess alcohol and the precipitated polysaccharide isolated as usual. A typical sample had the following properties: $[\alpha]_D^{18°} - 17°$ (c, 0·5); acid equivalent (after precipitation from HCl), 1000; ash, 4·6 %; moisture, 10 %; uronic anhydride, 18·0 %; nitrogen (traces).

On hydrolysis the rotation changed from $[\alpha]_D - 15°$ to $+22°$ (when heated with N H_2SO_4 at 80° for 2 hr.). Crystalline *d*-glucose ($[\alpha]_D + 52·5°$) was isolated in 67 % yield together with the barium salt of a uronic acid in 23 % yield (Ba, 16 %). This salt is being further investigated and may be an aldobionate.

Polysaccharide from the lucerne strain of Rhizobium radicicolum

According to Hopkins *et al.* [1930] the gum from the *Rh. radicicolum* (lucerne strain) contains much less glucuronic acid (4 %) than that from the clover strain which has (approx.) 20 % glucuronic acid. As we had available an improved culture medium, it was thought desirable to isolate the polysaccharide from the lucerne strain in order to ascertain whether, under conditions more advantageous to growth and metabolic activity of the organism, the polysaccharide would contain a higher percentage of uronic acid. Our observations confirmed those of Hopkins *et al.* in that "lucerne" polysaccharide from agar medium containing glucose had 4·4 % uronic anhydride and the "lucerne polysaccharide from agar medium containing sorbitol had 3·6 % uronic anhydride. Investigations are in progress to determine whether each different strain of the *Rh. radicicolum* produces a polysaccharide specific to that strain.

II. *Polysaccharide formation by* Azotobacter chroococcum

On liquid media. The organism was freshly isolated from soil at Edgbaston, Birmingham. It was particularly active as a gum-producer, for its synthetic powers did not diminish over a long period of subculturing. Investigation of polysaccharide formation in liquid media was continued and the properties of the materials isolated were compared with those of the polysaccharide grown on a solid medium. After a series of experiments during which the amounts and nature of both carbon and nitrogen sources were varied considerably, the following medium was found convenient and gave optimum yields: K_2HPO_4 0·5 g./l., $MgSO_4$, $7H_2O$ 0·2 g./l., NaCl 0·2 g./l., $CaSO_4$ 0·1 g./l., $FeCl_3$ (trace), sucrose 50·0 g./l., peptone 1·0 g./l., $CaCO_3$ 20 g./l.

Polysaccharide formation was poor when large volumes of medium were used and our results confirmed Anderson's observation [1933] that when growth did take place it was always accompanied by gum formation. Sorbitol (50 g./l.) was a good alternative to sucrose and in this case the metabolism solutions became highly viscous. The use of sorbitol minimizes any possible contamination from "laevan-forming" organisms [Cooper & Preston, 1935]. Yield of crude polysaccharide, 0·3 g./l. The products formed viscous suspensions in water which were too opaque for observation of optical rotation and very scanty amounts were obtained after purification. The medium was eventually abandoned.

On a gelatin medium. The medium used was essentially the same as that described for production of *Rh. radicicolum* polysaccharide. The method of growth, isolation and purification of the polysaccharide was also similar.

Properties of the polysaccharide. $[\alpha]_D - 2°$ (*c*, 0·52) in water; ash, 3·1 %. Hydrolysis with $N/2$ H_2SO_4 (*c*, 0·52), $[\alpha]_D - 2°$ (initial value), 0° (30 min.), +6° (70 min.), +10° (190 min.), +16° (280 min.), +20° (355 min.), +24° (690 min.) (equilibrium value). A naphthoresorcinol test for uronic acid was positive.

On an agar medium. Detailed accounts of the numerous investigations carried out in order to determine the best condition of growth are unnecessary. A medium of the following composition gave reliable results: (dissolved in 1 l. of 10 % yeast water) K_2HPO_4 0·8 g., KH_2PO_4 0·2 g., NaCl 0·2 g., $MgSO_4$, $7H_2O$ 0·2 g., $CaSO_4$ 0·1 g., $FeSO_4$ 0·01 g., $CaCO_3$ 5·0 g., sucrose 50 g., agar 17 g.

The medium was distributed in 200 ml. portions in Roux bottles, sterilized by steaming on three successive days and inoculated (using sterile pipettes) from an emulsion of a 48 hr. fluid culture. After incubation for 10 days at 25° the white viscous growth was washed off with a small amount of distilled water and the solution filtered through cotton wool into excess alcohol. The polysaccharide was readily precipitated in the form of a white fibrous mass which in appearance closely resembled the *Rh. radicicolum* polysaccharide, although the latter usually contained considerably larger quantities of bacterial protein. Purification of the polysaccharide was readily effected by filtration through kieselguhr in the manner previously described. The polysaccharide showed $[\alpha]_D - 2°$ (*c*, 0·52). It dissolved rapidly in water to give a viscid clear solution which was neutral. Ash, 8·3 %; uronic anhydride, 4 %. Hydrolysis was carried out at 100° with $N/2$ H_2SO_4 and the following rotational changes were observed: initial value $[\alpha]_D - 6°$ (*c*, 1·2), 60 min. −2°, 96 min. +2°, 138 min. +5°, 168 min. +7°, 210 min. +10°, 330 min. +17°, 510 min. +25°, 570 min. +26° (equilibrium value). From the hydrolysis solution crystalline glucose, $[\alpha]_D^{20°} + 52·3°$ (*c*, 1·0), was isolated in 87 % yield together with a small amount of the barium salt of a uronic acid (3 % yield) which gave a positive napthoresorcinol test. Tests for ketose or pentose were negative and no mucic acid could be isolated from oxidation of the hydrolysate.

Summary

Suitable media for large scale preparation of the polysaccharides from *Rhizobium radicicolum* (clover strain) and from *Azotobacter chroococcum* have been described. The polysaccharides have been purified and their hydrolysis products examined. This *Azotobacter* polysaccharide contains glucose units (about 90 %) and a small amount of a uronic acid residue (3–4 %). In this respect and in physical properties the polysaccharide resembles that from *Rh. radicicolum* and both probably belong to the same class as the specific polysaccharides of *Pneumococcus* types II and III.

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CCXXVIII. THE ISOLATION OF ANDROSTERONE AND *TRANS*DEHYDROANDROSTERONE FROM THE URINE OF NORMAL WOMEN

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It was first reported by Womack & Koch [1932] that the urine of normal women yielded quantities of androgenic material which, as measured by the capon test, were of the same order as that yielded by the urine of normal men. This fact has been confirmed repeatedly [cf. Siebke, 1934; Dingemanse *et al.* 1935; 1937; Simpson *et al.* 1936; Callow, 1936; Koch, 1936; Gallagher *et al.* 1937; Callow, 1938; Callow *et al.* 1938] and, with the improved methods of extraction which have been used during the last two years, it is generally recognized that, although the average amount of urinary androgen is somewhat lower for women than for men, the limits of normal variation for the two sexes overlap. Nevertheless, there has been no report of attempts to identify chemically the androgenic compounds in the urine of normal, non-pregnant women, parallel with the work by Butenandt and his collaborators [1934; 1935; 1937], which established that androsterone and *trans*dehydroandrosterone occurred in the urine of men, and indicated the presence of *iso*androsterone and of *epi*aetiocholanediol.

Work carried out in this laboratory [Callow *et al.* 1938] has shown that in respect of the ratio of methyleneketone, or "sterone" content (estimated colorimetrically) to comb-growth activity, no distinction can be made between urine extracts from men or women, but this rather vague evidence required amplification by more precise chemical analysis. With this end in view we undertook the more detailed fractionation of extracts of urine from both males and females. The concentration of androgenic activity in the neutral ketonic fraction of men's urine obtained by the use of Girard's reagent has been described in a previous paper [Callow *et al.* 1938]. When the ketonic resin was distilled at low pressure, volatile fractions were obtained, from the first of which the oxime of androsterone could be separated in the pure state in a yield of 0·2 mg. per l. of original urine. Parallel with these experiments, a concentrate from women's urine was put through the same process. There was a striking similarity in the behaviour of the two types of extract in the whole course of the fractionation, and this culminated in the isolation from the women's urine concentrate of androsterone oxime in a yield of 0·44 mg. per l. of original urine. The identity of the compound was confirmed by hydrolysis to androsterone and conversion of the latter into the benzoate. From a second fraction, distilling at a higher temperature, *trans*dehydroandrosterone was isolated in small amount as the characteristic benzoate.

Experimental

Collection and extraction of urine. Urine was collected from seven individual women patients in Middlesex Hospital. Their ages ranged from 20 to 35 years, and none had any obvious sexual dysfunction. Toluene was used as preservative,

and the collections from periods of 6–7 days, having *p*H values from 5·5 to 7, were hydrolysed and extracted as soon as they were available, using the method described by one of us [Callow, 1936]. The neutral fractions were made up in alcoholic solution and, after removal of sufficient material for colorimetric and capon assays, the solutions were combined and evaporated to dryness. The extract from 75 l. of urine or 76 days' output obtained in this way was dissolved in 150 ml. of hot methanol, kept overnight and filtered from a small amount of insoluble material. Evaporation of the filtrate yielded a residue weighing 2·59 g. A colorimetric assay by the modified Zimmermann method [Callow *et al.* 1938] gave a value of 8·2 mg. of methyleneketone or "sterone" per litre, i.e. the chromogenic equivalent of 0·615 g. of 17-ketosteroids in the whole sample.

Separation of ketonic fraction. The methanol-soluble fraction of the extract from 32 l. of urine, wt. 1·13 g., was dissolved in 20 ml. of glacial acetic acid, 2 g. of Girard's reagent T added, and the mixture heated on the water bath for 30 min. After cooling, it was poured into 400–500 ml. of ice and water containing enough Na_2CO_3 to neutralize, to phenolphthalein, 18 ml. of acetic acid. The mixture was extracted 7 times with 100 ml. lots of ether to remove the non-ketonic fraction. The combined ether extracts were washed once with water, which was added to the aqueous ketonic layer. The latter was acidified with 10 % of its volume of conc. HCl, warmed gently and kept standing for 1·5 hr. It was then extracted 5 times with 100 ml. lots of ether; the extract was washed with water, 0·5 *N* Na_2CO_3 and again with water, dried over Na_2SO_4 and evaporated. The residue weighed 423 mg. The ethereal solution of the non-ketonic fraction was washed, dried and evaporated. Wt. of residue, 487 mg. The ketonic fraction was dissolved in acetic acid and treated again with Girard's reagent. A ketonic fraction weighing 292·7 mg. was obtained. Colorimetry indicated a content of 75 % of "sterone".

Isolation of androsterone. The ketonic material, in alcoholic solution, was transferred to the vacuum still and evaporated to dryness. The still used consisted of a vertical, cylindrical tube with rounded bottom, of 4·5 cm. internal diameter, with a concentric water-cooled condenser of 3·5 cm. external diameter reaching to within 0·5 cm. of the bottom of the outer tube. Distillation was carried out at 0·0009 mm. and a bath temperature of 90–95° for 3 hr. 77·4 mg. of an almost colourless resin distilled. A colorimetric estimation gave a value of 80 % of "sterone".

72 mg. of the distillate, 40 mg. of hydroxylamine hydrochloride and 40 mg. of anhydrous sodium acetate were dissolved in about 10 ml. of alcohol and boiled under reflux for 5½ hr. The solution was filtered to remove NaCl, and evaporated to a volume of 2–3 ml. A few drops of water were added, and an oil separated, which crystallized on scratching. Next day the solid was collected, washed rapidly with a little cold 50 % aqueous alcohol and dried: yield, 21·6 mg., M.P. 149–194°.

After three recrystallizations from acetone, a product was obtained crystallizing in well-formed rhombic plates, M.P. 208–213·5° after softening at 204° (Kofler apparatus; see note below). The M.P. was unchanged by admixture with an authentic specimen of androsterone oxime (M.P. 210–211°).

14 mg. of once recrystallized oxime, M.P. 203–206°, were dissolved in 2 ml. alcohol, 1 ml. of 3 *N* H_2SO_4 added and the mixture boiled under reflux for 3 hr. The crude ketone, precipitated by water, weighed 3 mg.; M.P. 168·5–175°. It crystallized from methanol in thin plates, M.P. 178–181°, after subliming to needles. A mixture with an authentic specimen of androsterone (M.P. 184–185°) melted at 178–181·5°.

About 2·5 mg. of crude androsterone (recrystallized product and material obtained by evaporation of the mother liquors above) were dissolved in 0·5 ml. of pyridine, and 2 drops of benzoyl chloride added. The mixture was warmed on the water bath for 5 min., cooled and treated with water drop by drop. An oil separated, which was extracted with ether. The extract was washed with water, dilute HCl, $NaHCO_3$ solution and again with water, dried over Na_2SO_4 and evaporated. The residue was crystallized from 2 drops of methanol. The product, M.P. 166·5–171° (soft at 166°), was again recrystallized from methanol. It then had M.P. 172–175° (soft at 169°), and a mixture with an authentic specimen of androsterone benzoate (M.P. 170–174°) melted at 170–174·5° (soft at 168°).

Separation of trans*dehydroandrosterone benzoate.* 192 mg. of ketonic fraction (from 20 l. of urine) were distilled as described above at 90–95°/0·002 mm. for 2 hr. After removal of the distillate, the still was evacuated to 0·0017 mm., the oil bath heated slowly to 145° and then kept at 145–150° for 1½ hr. 84 mg. of distillate were obtained. Colorimetric assay indicated a content of 87% of "sterone". A comparative experiment showed that *trans*dehydroandrosterone slowly sublimed between 120 and 150° under the above conditions.

The distillate was dissolved in about 2 ml. of dry pyridine, treated with 3 drops of benzoyl chloride and the mixture warmed for 5 min. It was then cooled and treated with water. An oil separated which was well washed with water and dissolved in about 2 ml. of methanol. After leaving overnight in the refrigerator, 1–2 mg. of a crystalline solid separated, M.P. 223·5–243·5°. After recrystallization once from methanol and once from ethyl acetate, the M.P. was 246–248° (softening 245°). A mixture with an authentic specimen of *trans*-dehydroandrosterone benzoate (M.P. 245·5–250°) melted at 245–248·5°. A further crop of crystals separated from the original methanol mother liquors which after recrystallization melted at 238–248° (mixture with *trans*dehydroandrosterone benzoate, M.P. 237–252°).

All the melting points recorded were observed on a microscope slide on an electrically heated stage, using Kofler's micro-melting point apparatus. The temperatures recorded are those of the first appearance of liquid and of complete fusion.

Discussion

It is abundantly clear that complete chemical analysis of extracts of normal urine into their steroid components is a difficult matter. The processes of partition between solvents, distillation and crystallization, not ideal for quantitative work at the best of times, are not well adapted to the separation of closely related steroids. Our yields of androsterone amounted to about the equivalent of 0·2 and 0·4 mg. per litre from urines of men and women, respectively. The higher yield in the latter case is to be attributed to practice in the manipulation, and there are clear indications that extracts of urine from normal men or women contain similar amounts. We do not feel, however, that it is possible to estimate the amounts originally present more accurately than Butenandt and his collaborators [1935] did when they concluded that the amounts of androsterone and *trans*dehydroandrosterone actually present in extracts of men's urine were at least twice those separated in the pure state. With the available methods of separation we are, in fact, very far from the stage of being able to account, by isolation of recognizable derivatives, either for the whole androgenic activity, or for the colorimetrically determined methylene-ketone content of normal urine extracts.

The importance of the present work lies in its demonstration that androsterone and *trans*dehydroandrosterone are not specific products of the male

organism. It is possible, however, that the excretion of identical androstane derivatives by the two sexes is simply due to these products being common degradation products, particularly adapted for excretion, of original secretions which, although different, are closely related in chemical structure. The alternative assumption of actual identity of the original secretions in the two sexes is more speculative, and could only apply to a part of the endocrine steroids, since the nature of only a fraction of the excretory transformation products is established. It has, however, some plausibility as regards products from the suprarenal glands, for evidence from suprarenal tumour cases leads to the belief that *trans*dehydroandrosterone at least is metabolically derived from the family of steroid compounds present in the suprarenal glands, and that its excretion is not directly connected with gonadal function (cf. the discussion by Callow [1938]). The degree of direct responsibility of the gonads for any part of the urinary excretion of androstane derivatives remains to be investigated; at present there is no clue to products which can be recognized as derived from the testis or the ovary, and any characteristic difference in the androstane derivatives excreted by the two sexes still eludes detection. It is hoped that more light on these problems will be obtained as chemical and physical methods are developed and applied to the more detailed analysis of urines from abnormal clinical types of patient. Work along these lines is now in progress.

Summary

Androsterone and *trans*dehydroandrosterone have been isolated from extracts of the urine of normal women, in a yield comparable with that from normal men's urine.

We wish to thank Prof. E. C. Dodds for arranging the collection of urine, the staff of the Courtauld Ward of the Middlesex Hospital for the trouble and care they have taken in this matter, and Mr S. W. Stroud, working in the Courtauld Institute of Biochemistry, for carrying out the first extraction of the urines from individual patients.

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CCXXIX. ELIMINATION OF AMINES IN MAN

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(*Received 30 August 1938*)

THE amine oxidase of mammalian tissues has been shown to oxidize adrenaline and a number of other amines to the corresponding aldehydes [Richter, 1937; Blaschko *et al.* 1937]. Of the 67 amines hitherto tested those of the general type $R.CH_2NH_2$ were readily oxidized, but amines of the type $RR'CH.NH_2$ showed no appreciable oxidation. The latter group includes the amines of the ephedrine series which are of particular interest owing to their selective action on certain parts of the nervous system and experiments were therefore carried out to determine what happens to them in the animal body and how they are eliminated.

Indications have been recorded in the literature that the elimination of amines may in some cases take a different course in laboratory animals from that followed in man. Slotta & Müller [1936] found for example that mescaline is oxidized in dogs and rabbits to the corresponding acid which they isolated from the urine, but they were unable to detect this acid in the urine in man.

In the present experiments a series of amines were given by mouth or intravenously to male adults of 60–75 kg. and believed to be normal. In agreement with the previous work with the isolated amine oxidase, amines of the type $R.CH_2NH_2$ were rapidly destroyed *in vivo* while amines of the ephedrine series were found to be excreted in the urine unchanged.

With benzedrine and methylisomyn the non-toxic dose is of the order of 10 or 20 mg., and since the excretion extended over 2 days the resulting concentrations in the urine were too small to be estimated by the ordinary methods available; a sensitive micro-method for estimating amines was therefore worked out in order to follow the elimination quantitatively.

Micro-estimation of amines

The method depends on the fact that picric acid gives little colour while amine picrates are strongly coloured in 50% chloroform-toluene or chloroform-light petroleum solution: it is generally applicable to the higher amines and alkaloids which are extracted from aqueous solution by organic solvents [Richter, 1938]. 60 ml. of urine in a 250 ml. separating funnel were made alkaline with 4 ml. 2*N* NaOH and shaken with 6 ml. toluene, using a rotating motion to avoid froth formation. The toluene layer was run into a centrifuge cup, centrifuged for 5 min., cleared by stirring with a glass rod and centrifuged again. 0·1 ml. of 2% picric acid in chloroform was added to 3 ml. of the toluene solution and 3 ml. of chloroform in a dry test tube and the mixture was kept for 12 hr. The clear yellow solution of the amine picrate was then compared colorimetrically with solutions prepared in a similar manner from standard amine solutions.

By this method concentrations as low as 0·5μg./ml. of β-phenylethylamine could easily be estimated. The urines were collected in bottles containing toluene to prevent amine formation by bacterial action. Normal urines were generally found to contain traces of amines coming apparently from the food (Lintzel [1934] observed an increased excretion of amines after feeding fish); but by giving a diet consisting chiefly of bread and butter the normal amine content of

the urine could be reduced to a value corresponding to 0–0·5 μg./ml. of phenylethylamine. Of the basic substances commonly present in urine which might be expected to interfere ammonia, methylamine, trimethylamine, trimethylamine oxide, indole and skatole at concentrations of 20 μg./ml. gave less colour than 0·5 μg./ml. of β-phenylethylamine and could be neglected. Bain [1914] claimed to have isolated *iso*amylamine from urine and that, if present, would interfere; but Guggenheim & Löffler [1915] were unable to confirm the presence of *iso*amylamine in normal urine and concluded that it is formed only by subsequent bacterial action on standing. No interference by *iso*amylamine was observed under the conditions of the present experiments.

Ephedrine. $C_6H_5CHOH.CH(CH_3)NH(CH_3)$

(*a*) *Identification.* Urine was collected for 12 hr. after feeding ephedrine hydrochloride (104 mg. base). The urine was made alkaline with NaOH, extracted with ether and the ethereal extract shaken with a small volume of *N*/10 HCl. On evaporation and recrystallization of the residue a hydrochloride was obtained which crystallized in colourless prisms showing parallel extinction and strong interference in polarized light, crystal edge angle 130° and M.P. 215–217°. Ephedrine hydrochloride crystals showed similar optical properties, crystal edge angle 131° and M.P. 217–219°. The mixture melted at 216–218°.

(*b*) *Rate of excretion.* Urine was collected every 3 to 4 hr. before and after giving ephedrine hydrochloride by mouth and the ephedrine in the urine was estimated by the micro-method described. The rate of excretion was maximal

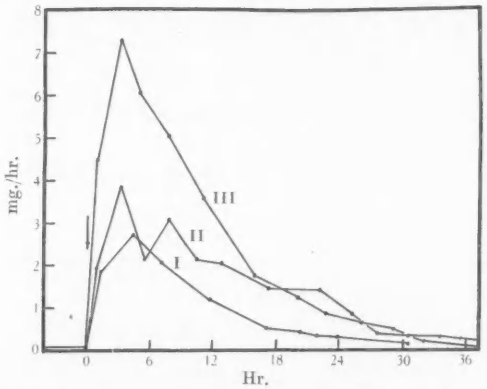

Fig. 1. Excretion of ephedrine in the urine. I, 26 mg. base; II, 52 mg. base; III, 104 mg. base. Given as the hydrochloride by mouth.

(115 μg./ml.) at about 4 hr. after administration, but with a large dose of 104 mg. a slow excretion at the rate of 0·25 mg./hr. could still be detected after 36 hr. The figures obtained for the fractions excreted at different times after administration were as follows:

Ephedrine (mg. base)	Time after administration 12 hr. %	24 hr. %	48 hr. %
26	77	97	97
52	59	92	99
104	59	77	82

These figures show that ephedrine is not appreciably oxidized in the body, but approximately 100% may be excreted unchanged in the urine. The lower value of 82% excretion obtained with 104 mg. ephedrine suggests that other methods of elimination, as in the sweat or faeces, may become significant when large amounts are given.

Benzedrine. $C_6H_5CH_2CH(CH_3)NH_2$

(*a*) *Identification.* Urine collected for 12 hr. after giving benzedrine sulphate (14·7 mg. base) by mouth was made alkaline with NaOH and extracted with toluene. The toluene solution was shaken with 10 ml. 1% HCl and the aqueous solution evaporated to dryness. The residue was transferred to a Conway dish and the picrate, picrolonate and 2 : 4-dinitronaphthol derivative were prepared by allowing the base to volatilize in the presence of traces of the reagents on microscope coverslips [Richter, 1937]. The picrate crystallized in yellow prisms showing parallel extinction and strong interference colours in polarized light and crystal edge angles 107, 124 and 134°. Benzedrine picrate crystallized in similar habit and gave similar optical properties and crystal edge angles 106, 124 and 133°. The picrolonates and dinitronaphthol derivatives also agreed in crystal habit and optical properties: the pleochroism of the picrolonates was very characteristic.

(*b*) *Rate of excretion.* 20 mg. benzedrine sulphate (14·7 mg. base) were given by mouth and the urinary amines were estimated before and after administration, using light petroleum for the extraction. The rate of excretion was slower than for ephedrine and it was confirmed in a number of experiments that benzedrine could still be detected in the urine for 36 hr. after administration. With 14·7 mg. base the fraction excreted in the urine in 24 hr. was 40% and in 48 hr. 70%.

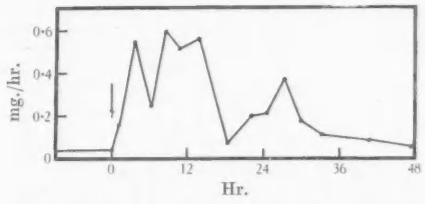

Fig. 2. Excretion of benzedrine (14·7 mg. base).

These figures make it unlikely that benzedrine is oxidized to any appreciable extent in the tissues. If the blood volume is taken as 7 l. the concentration in the blood for this dose would be at the most 2·1 μg./ml. and is probably much lower than this since the base is lipoid-soluble and is known to enter the nervous system. The concentration of benzedrine found in the urine was 10–13 μg./ml. a few hours after administration so that the kidneys must effect considerable concentration of the amine.

Methylisomyn. $C_6H_5CH_2CH(CH_3)NH(CH_3)$

Methylisomyn hydrochloride (8 mg. base) was given by mouth. As for benzedrine the rate of excretion was slow, being significant at 48 hr. after administration, and the recovery in the urine was 36% in 24 hr. and 56% in

48 hr. The maximal concentration found in the urine was 5 μg./ml. which again shows a considerable concentration by the kidneys such as was also found with ephedrine and benzedrine.

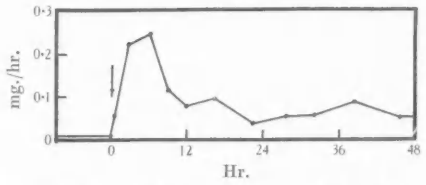

Fig. 3. Excretion of methylisomyn (8 mg. base).

Amines of the type $R.CH_2NH_2$

The following amines were given by mouth in the form of the hydrochlorides: *iso*amylamine (100 mg. base), benzylamine (160 mg. base), β-hydroxy-β-phenylethylamine (50 mg. base) and β-phenylethylamine (300 mg. base). Although the experimental method was sufficiently sensitive to detect 1 μg./ml. of amine no increased amine excretion was observed after administration.

Attempts were then made to show the rate of elimination of β-phenylethylamine by looking for the corresponding phenylacetic acid in the urine: Guggenheim & Löffler [1915] showed that phenylacetic acid is formed from β-phenylethylamine in the rabbit.

Identification of phenylacetic acid. Urine voided after feeding β-phenylethylamine hydrochloride (300 mg. base) was heated for 3 hr. at 100° with 0·1 vol. of conc. H_2SO_4 to decompose phenylacetylglutamine [Thierfelder & Sherwin, 1914]. The acids obtained by extracting with toluene were separated by fractional crystallization and consisted mainly of benzoic acid and an acid which crystallized from water in triangular or hexagonal plates giving crystal edge angle 128° and having a characteristic smell. Phenylacetic acid crystallized in similar habit and gave the edge angle 128° and the same smell.

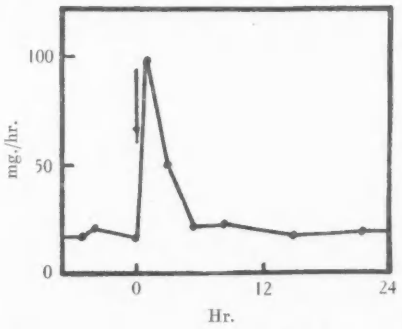

Fig. 4. Excretion of phenylacetic acid after taking β-phenylethylamine (300 mg. base) by mouth.

Rate of excretion of phenylacetic acid. The normal excretion of benzoic acid was reduced to a minimum by keeping on a diet free from fruit and vegetables.

Urine specimens collected before and after feeding β-phenylethylamine hydrochloride (300 mg. base) were heated for 3 hr. at 100° with 0·1 vol. conc. H_2SO_4. 100 ml. samples of the urine were then shaken with 10 ml. of toluene and the toluene-soluble acids were titrated with $N/10$ NaOH using thymol blue. A partition curve was then prepared by treating standard solutions of phenylacetic acid in a similar manner and so the concentration of toluene-soluble acid in the urine, taken as phenylacetic acid, was estimated. No information is available as to the rate of conjugation of phenylacetic acid with glutamine to form the phenylacetylglutamine which is excreted, but in the present experiments the increased excretion of toluene-soluble acid corresponded to 62 % of the theoretical for 300 mg. β-phenylethylamine in $4\frac{1}{2}$ hr.: the oxidation of the amine must therefore be comparatively rapid.

Mescaline. $(OCH_3)_3C_6H_2CH_2CH_2NH_2$

(*a*) *Identification.* Mescaline hydrochloride (191 mg. base) was given intravenously and the urine was made alkaline and extracted with toluene. The toluene solution was concentrated and treated with a solution of picric acid in chloroform. The picrate which crystallized out on standing formed diamond-shaped prisms giving an extinction angle of 10° in polarized light, crystal edge angle 141° and M.P. 221–223°. Mescaline picrate crystallized in similar habit and gave similar optical properties, extinction angle 10°, edge angle 142° and M.P. 221–223°. The mixture showed no depression of M.P. Mescaline was also identified in the same way in the urine after giving by mouth.

(*b*) *Rate of excretion.* When mescaline hydrochloride (191 mg. base) was given intravenously the fractional excretion in the urine was 52 % in 24 hr. and with the same dose given by mouth 58 % in 24 hr. The main bulk of the mescaline

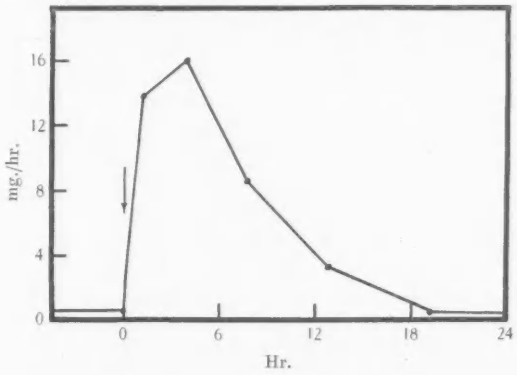

Fig. 5. Excretion of mescaline after taking 191 mg. base as the hydrochloride by mouth.

is clearly excreted unchanged, but the amount found in the urine falls considerably short of 100 % which suggests that the body may have a certain limited capacity for oxidizing mescaline or eliminating it in some other way.

Discussion

The work of Ewins & Laidlaw [1910] and Guggenheim & Löffler [1915] led to the general conclusion that amines are rapidly eliminated by oxidation in the animal body. On the other hand there is pharmacological evidence that amines may in some cases remain active in the body for a considerable length of time. The action of ephedrine for example is known to continue for many hours and the effects of benzedrine have been observed to persist for more than a day [Guttmann & Sargant, 1937]. It has always remained doubtful, however, how far the various effects observed were due to the direct action of the amines and how far they were after-effects due to tissue damage caused by the amines: this could be decided only by accurate information as to the rate of elimination.

The present experiments *in vivo* confirm the previous observations on the significance of the amine oxidase since those amines which are oxidized by the enzyme (type $R.CH_2NH_2$) were also destroyed in the body, while amines of the ephedrine series (type $RR'CH.NH_2$) which are not oxidized by the enzyme were slowly excreted in the urine from which they could be recovered unchanged. The pharmacological data as to the persistent action of amines of the ephedrine series can therefore be explained in terms of the specificity of the amine oxidase.

With amines that are oxidized in the body the efficiency of the inactivation *in vivo* is shown in the case of β-phenylethylamine by the promptness with which phenylacetic acid appears in the urine and by the fact that even with doses of 300 mg. no amine excretion could be detected in the urine.

Mescaline, which is a phenylethylamine derivative, might be expected to be oxidized by the amine oxidase, but Blaschko *et al.* [1937] found little oxidation of mescaline by preparations of guinea-pig amine oxidase and it would appear that the bulky methoxyl groups modify the molecule so that it is able to resist oxidation. Slotta & Müller [1936] found that mescaline is completely oxidized *in vivo* in the rabbit and dog, but Bernheim & Bernheim [1938] concluded that some factor other than the amine oxidase is responsible for the oxidation. Whatever this factor may be it is clearly deficient in man and the case of mescaline illustrates the fact that with drugs of this type the behaviour in one kind of animal may be quite different from that in another.

Summary

1. A sensitive micro-method for estimating amines has been used for studying the elimination of amines in man.

2. Amines of the ephedrine series and also mescaline are excreted unchanged and were isolated from the urine in the form of crystalline derivatives.

3. Amines of the general type $R.CH_2NH_2$ are rapidly oxidized in man.

4. The rates of excretion of ephedrine, benzedrine, methylisomyn and mescaline have been measured.

The author wishes to thank Prof. F. Golla for his interest and advice, Dr Blackburn, Dr Hill and Dr Guttmann of the Maudsley Hospital for their co-operation in administering or taking the drugs, and Messrs Burroughs and Wellcome for the gift of *l*-methylisomyn.

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CCXXX. THE USE OF COMPOUNDS RELATED TO *p*-AMINOBENZENESULPHONAMIDE IN THE TREATMENT OF CERTAIN INFECTIONS IN MICE

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(*Received 27 August 1938*)

It is now well established that *p*-aminobenzenesulphonamide has some effect on septicaemias in mice other than those caused by the haemolytic *Streptococcus* [Buttle *et al.* 1936; Proom, 1937; Buttle *et al.* 1937 etc.], and further that various related compounds exert chemotherapeutic action in certain mouse infections. However, in no case has it been shown that the *in vivo* effects run parallel with the *in vitro* results, and the mode of action is still very obscure.

The object of the present investigation has been to compare the chemotherapeutic actions of a number of selected compounds on mice infected with various pathogenic bacteria. The organisms chosen were the *Streptococcus*, the *Staphylococcus*, the *Pneumococcus* and *Bacillus aertrycke*. At the same time experiments were carried out to determine what action, if any, the compounds exerted on the growth of these bacteria *in vitro* in order to ascertain whether the action *in vitro* could in any way be correlated with the chemotherapeutic activity as exhibited in animal experiments.

In vivo *experiments*

A. *Infection of animals.* Using the mouse as the experimental animal approximately 100 M.L.D. of the infecting organism were injected intraperitoneally. The inoculum was obtained from an 18-hr. slope culture which was emulsified and made up to the required dilution with either sterile saline or 5% mucin. Some organisms were not sufficiently virulent to kill mice when suspended in saline, and so a method involving the use of mucin similar to that of Miller & Castles [1936] was used to diminish the minimum fatal dose of these bacteria. 1 ml. of the bacterial dilution was injected intraperitoneally into mice of 20–25 g. weight.

B. *Chemotherapeutic treatment.* The various compounds under investigation were first tested for their toxicity when administered to normal mice orally through a feeding tube. Whenever possible the compound was brought into solution in a suitable solvent, but otherwise it was given in the form of a suspension in gum acacia. The doses given to the infected mice varied according to the toxicity but in each case the drug was fed immediately after the injection of organisms and at intervals thereafter. The frequency of feeding varied according to the reaction of the animal to the organism and to the drug.

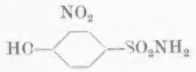
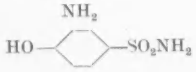
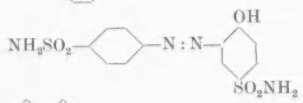
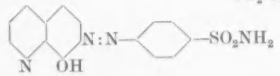
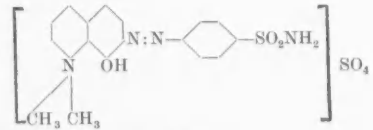
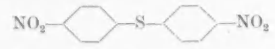
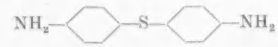
The non-toxic dose employed for the majority of compounds was 10 mg. administered by mouth twice daily for 2–3 days.

Results of animal experiments. The results obtained from experiments with a series of compounds are shown in Table I. In the case of *p*-aminobenzenesulphonamide, with the *Streptococcus* as the infecting organism, the usual clear-cut

[1] Carnegie Research Scholar.

Table I. In vivo *experiments*

100 M.L.D. of infecting organisms injected intraperitoneally into mice of 20–25 g.
10 mg. therapeutic agent fed immediately, after 5 hr. and twice daily for 2–3 days.

Compound	Formula	Oral toxicity for 20 g. mouse mg.	Solvent	Therapeutic activity			
				Strepto-coccus	*Pneumo-coccus*	*Staphylo-coccus*	*Bacillus aertrycke*
p-Aminobenzenesulphonamide		80	Water	+ + +	+ to ±	±	±
p-Hydroxybenzenesulphonamide		50-60	Water	± to 0	±	±	0
3-Nitro-4-hydroxybenzene-sulphonamide		>100	Sodium bi-carbonate	+ +	±	0	0
3-Amino-4-hydroxybenzene-sulphonamide		80	Suspension in gum acacia	±	±	±	0
p-Aminobenzenesulphonamide coupled with *p*-hydroxyben-zenesulphonamide		20	Water	±	±	0	0
p-Aminobenzenesulphonamide coupled with 8-hydroxyquino-line		20	Suspension in gum acacia	± to 0	0	±	0
p-Aminobenzenesulphonamide coupled with 8-hydroxyquino-linemethosulphate		60	Water	± to 0	0	±	0
4:4′-Dinitrodiphenylsulphide		>100	Suspension in gum acacia	0	±	0	0
4:4′-Diaminodiphenylsulphide		About 60	Lactic acid	±	±	0	0
4:4′-Diacetyldiaminodiphenyl-sulphide		>100	Suspension in gum acacia	±	±	+ to ±	0

+ + + 100% recovery, + + 75% recovery, + 25–30% recovery, ± death delayed, 0 no effect.

evidence of chemotherapeutic activity was obtained, 100% of the animals infected recovering completely within 3–4 days. With the other compounds the best result was obtained with 3-nitro-4-hydroxybenzenesulphonamide when, in a series of haemolytic streptococcal experiments, 75% of the treated animals survived indefinitely. This compound also showed slight action against the pneumococcal infection of mice but there was no effect on the *Staphylococcus* or on *Bacillus aertrycke*. As the toxicity of this compound is less than that of *p*-aminobenzenesulphonamide its use as a therapeutic agent is worthy of consideration.

Of the other results the most important is the antistaphylococcal action of diacetyldiaminodiphenylsulphide.

In vitro *experiments*

Technique. When possible, solutions of the compounds were prepared; otherwise fine suspensions were employed. In every case the *p*H was adjusted to 7·6. Dilutions were made in broth or serum broth according to the organism to be tested. These broth tubes were inoculated with 0·1 ml. of a thick bacterial suspension obtained by emulsifying an 18-hr. slope culture of the organism, and incubated at 37° for 18 hr. In order to determine both the bacteriostatic and the bactericidal activities of the compound, subcultures from the broth tubes were made on blood-agar plates after 3, 6 and 18 hr. incubation, and from the growth readings of these plates the activity of the drug was ascertained. The results obtained with the series of compounds used in the *in vivo* experiments is shown in Table II.

Table II. In vitro *experiments*

Each papain broth tube was inoculated with 0·1 ml. of a thick suspension of organisms, *p*H 7·6, *r*H 19–20. Subcultures were made after 3, 6 and 18 hr., and from these readings the bacteriostatic and bactericidal activities were estimated.

Compound	Bactericidal and bacteriostatic activities: *Streptococcus*	*Pneumococcus*	*Staphylococcus*	*B. aertrycke*
p-Aminobenzenesulphonamide	+ + +	+ + +	0	± to 0
p-Hydroxybenzenesulphonamide	+ + +	+ + + +	0	0
3-Nitro-4-hydroxybenzenesulphonamide	+ + + +	+ + +	0	0
3-Amino-4-hydroxybenzenesulphonamide	+ +	+ + +	0	0
p-Aminobenzenesulphonamide coupled with *p*-hydroxybenzenesulphonamide	+ +	+	± to 0	0
p-Aminobenzenesulphonamide coupled with 8-hydroxyquinoline	+ + to +	+ + + +	+	±
p-Aminobenzenesulphonamide coupled with 8-hydroxyquinolinemethosulphate	+ + to +	+ + + +	+	±
4:4′-Dinitrodiphenylsulphide	+ +	+ +	±	0
4:4′-Diaminodiphenylsulphide	+ + to +	+ +	±	0
4:4′-Diacetyldiaminodiphenylsulphide	+ + to +	+ +	±	+ to ±

+ + + + Bacteriostatic at 1 in 10,000, bactericidal at 1 in 1000.
+ + + ,, 1 in 10,000, ,, 1 in 100.
+ + ,, 1 in 1000, ,, 1 in 10.
+ ,, 1 in 100.
± ,, 1 in 10.
0 No effect.

Results of in vitro *experiments.* The bactericidal action of these compounds on the *Pneumococcus* is greater than is to be expected from the results of animal experiments. Though this is partly to be explained by the low viability of the *Pneumococcus* as compared with other organisms, the difference between the

antipneumococcal activities of *p*-hydroxybenzenesulphonamide and the quinoline derivatives in the test tube and in the living animal requires further investigation. Although many compounds, as is shown in Table II, are bactericidal to the *Pneumococcus*, yet in no case has it been possible to cure mice with a pneumococcal septicaemia.

The results with the *Streptococcus* in one or two cases show a slight parallelism with those obtained in animal experiments, but with the majority of compounds the antiseptic value shows little correlation with the curative power.

Apart from causing a slight delay in the growth rate in a few experiments, the compounds give almost uniformly negative results with the *Staphylococcus* and *Bacillus aertrycke*.

Discussion and conclusions

Perhaps the most interesting result of the work here reported is the relatively high antistreptococcal activity of 3-nitro-4-hydroxybenzenesulphonamide. All the sulphur-containing compounds previously found to exercise marked chemotherapeutic activity against septicaemias caused by the haemolytic *Streptococcus* have an amino group or a group which can be easily converted into the amino group, e.g. the nitro group, either free or substituted in the *p*-position in the benzene ring. The activity of the new compound indicates that this rule is not general and suggests that it is important to prepare and test other derivatives possessing the 3-nitro-4-hydroxybenzene grouping. It will be noted that 3-nitro-4-hydroxybenzenesulphonamide is definitely less toxic than sulphanilamide. This compound was also active on pneumococcal septicaemias in mice but in this case its efficacy was definitely inferior to that of *p*-aminobenzenesulphonamide.

p-Hydroxybenzenesulphonamide, as well as certain disubstituted benzenesulphonamides, have been tested by Tréfouel *et al.* [1937] in respect of their antistreptococcal activity in mice, in every case with practically negative results. Our results with the first of these compounds are in agreement with their findings; of the disubstituted derivatives used by Tréfouel *et al.* none were available to us.

The antistaphylococcal activity of 4:4′-diacetyldiaminodiphenylsulphide is of some interest. Though the activity of this compound against the *Staphylococcus* is not very great, under favourable conditions it does delay death by about 4 days, and this indicates that antistaphylococcal action is not limited to certain sulphonamide derivatives but belongs also to other classes of sulphur-containing compounds.

The main conclusion to be drawn from a comparison of the experiments carried out on infected mice and those performed *in vitro* is a negative one. There is no obvious connexion, as far as our results show, between antibacterial actions under these two different conditions. Thus it would appear that the effect of the compound on the bacteria which is of importance for its chemotherapeutic action is not one of general toxicity, i.e. a simple inhibition of growth or metabolism, but is rather a highly specific and characteristic one. The view that this group of active sulphur-containing compounds exercise their chemotherapeutic action through some special damaging effect on the resistance of the organisms to the antibacterial action of the blood and the body tissues has been advocated by many investigators, and has recently received support from the observations of Whitby [1938]. He has shown that the remarkable chemotherapeutic action of 2-*p*-aminobenzenesulphonamidopyridine on mice infected with pneumococci is associated with the loss by the bacteria of their capsule which makes the organisms much more susceptible to phagocytosis and other similar processes. The balance of the evidence seems to suggest that some similar though perhaps

more subtle change is effected in organisms such as the *Streptococcus* by compounds of the sulphonamide group and other active sulphur-containing derivatives.

SUMMARY

1. Ten compounds have been tested in respect of their chemotherapeutic actions on mice infected with haemolytic streptococci, pneumococci, staphylococci or *Bacillus aertrycke*. The antibacterial actions *in vitro* of these compounds have also been examined.

2. 3-Nitro-4-hydroxybenzenesulphonamide has a marked curative effect on streptococcal septicaemias in mice. The low toxicity of this compound suggests that it should be investigated on a wider scale.

3. Diacetyldiaminodiphenylsulphide delays the death of mice infected with staphylococci.

4. Under the conditions of experimentation, no correlation could be established between the action of these compounds *in vitro* and their chemotherapeutic activity on infected mice. This result is in accordance with the view that the important action of these compounds on the organisms is not one of gross toxicity, but that it involves a damaging of the defences which normally protect the organisms from the bactericidal processes of the body.

I wish to express my thanks to Dr W. O. Kermack for his help and encouragement during the course of this work, and for supplying me with the compounds tested.

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CCXXXI. THE COMPONENT GLYCERIDES OF AN OX DEPOT FAT

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The general composition of the depot fats of the ox and the pig was indicated by Banks & Hilditch [1931; 1932] by consideration of the proportion of fully saturated glycerides present in these fats together with the percentage composition of the fatty acids in the whole fats and in their fully saturated glycerides. Later, in the case of a pig depot fat, Hilditch & Stainsby [1935] obtained somewhat more detailed information by its study after it had been progressively hydrogenated to varying extents, coupled with the determination of tristearin in the completely hydrogenated fat. More recently it has been found possible to obtain an approximate estimate of at all events the main component glycerides present, in solid fats of comparatively simple fatty acid composition, by preliminary systematic crystallization of the fat from acetone. This procedure, although usually incapable of yielding definite individual mixed glycerides, causes a division of the fat into sparingly soluble portions in which mono-unsaturated-disaturated (and fully saturated) glycerides predominate, and more soluble portions in which the di-unsaturated glycerides (and tri-unsaturated glycerides when present) are concentrated. The fat is thus divided into two or three fractions, each of which is investigated as follows:

(*a*) the component acids are determined by ester-fractionation;

(*b*) a portion is hydrogenated and the tristearin content of the product determined;

(*c*) where necessary, the fully saturated glycerides present are isolated and determined; the component acids present in the fully saturated glycerides, and the tristearin content of the latter, are determined.

In addition to the data for fully saturated glycerides, it is then possible, in each fraction of the fat, (i) to estimate the proportions of mono- and of di-unsaturated glycerides (or of di- and tri-unsaturated glycerides) and (ii), knowing the tri-C_{18} glyceride content (determined as tristearin in (*b*)), to estimate the proportions of mono-C_{18}- and di-C_{18}-mixed glycerides in which another homologous acid (palmitic) is present. With these data, and taking into account the known general order of solubility in acetone of, for example, oleodistearin, dioleostearin, oleopalmitostearin, oleodipalmitin and palmitodiolein, it is usually possible to give a detailed, approximately quantitative statement of the component glycerides in each portion of the fat, and therefrom to deduce that of the whole fat.

This method has been used in the investigation of a number of solid seed fats, including cacao butter, mowrah fat and shea fat, by Hilditch *et al.* [1936; 1938, 1, 2, 3]. The present communication describes its application to a typical ox depot fat. Here the procedure is somewhat more complicated owing to the comparatively large proportions of fully saturated glycerides present, and to the circumstance that animal depot fats contain a somewhat greater number of minor component acids than any of the seed fats to which the method has yet been applied.

Experimental

The ox depot fat employed was obtained by extracting with acetone the perinephric tissue from a Shorthorn heifer. It had I.V. (Wijs) 38·7, sap. equiv. 284·0, and contained 0·12% of unsaponifiable matter. The fatty acids (185·9 g.) obtained on hydrolysis of 200 g. of the fat were submitted to lead salt separation by the modified Twitchell method [Banks *et al.* 1933], and the resulting acid fractions were converted into methyl esters and fractionally distilled. The esters of the "solid" acids were distilled from a Willstätter bulb; those of the "liquid" acids were distilled through the electrically heated and packed column described by Longenecker [1937]. The final results of the analysis are summarized in Table I.

Table I. *Component acids of ox depot fat*

Component acids	"Solid" (56·6%)	"Liquid" (43·4%)	Total	% (wt.)	% (mol.)
Saturated:					
Lauric	—	0·50	0·50	0·5	0·7
Myristic	0·68	2·04	2·72	2·7	3·2
Palmitic	27·17	3·23	30·40	30·4	32·2
Stearic	23·63	—	23·63	23·7	22·6
Unsaturated:					
Tetradecenoic	—	0·41	0·41	0·4	0·5
Hexadecenoic	—	1·65	1·65	1·7	1·8
Oleic	5·08	33·49	38·57	38·6	37·1
Octadecadienoic	—	2·00	2·00	2·0	1·9
Unsaponifiable:	0·04	0·08	0·12	—	—

Crystallization of the ox depot fat from acetone

About 1 kg. of the fat was subjected to systematic crystallization from acetone at 0° or room temperature as follows.

(i) The fat (in three portions for convenience) was dissolved in acetone (5 ml. per g. of fat) and kept at room temperature overnight; after filtering the separated fat the solution was then cooled at 0° overnight and a further fraction of fat separated:

Fat taken	Separated at room temperature			Separated at 0°			Left in solution at 0°		
g.	No.	g.	I.V.	No.	g.	I.V.	No.	g.	I.V.
350	A_1	102	22·8	B_1	130	35·7	C_1	118	56·3
350	A_2	95	16·9	B_2	161	42·2	C_2	94	57·6
325	A_3	96	19·3	B_3	142	41·6	C_3	87	57·6

(ii) Corresponding fractions were then united and further crystallized as shown in the scheme below:

Fractions crystallized		Conditions of crystallization			Separated fat			Fat left in solution		
Nos.	g.	Acetone (ml. per g. fat)	Temp.	Time (hr.)	No.	g.	I.V.	No.	g.	I.V.
$A_1+A_2+A_3$	293	3 : 1	Atmospheric	16	A_4	244	15·9	A_5	49	41·1
$B_1+B_2+B_3$	433	5 : 1	0°	6	B_4	347	37·1	(further 16 hr. at 0°)		
			0°	16	B_5	27	40·4	B_6	59	55·3
A_5+B_5	76	5 : 1	0°	6	D_1	66	40·6	D_2	10	53·8

The eight fractions (A_4, B_4, B_6, D_1, D_2, C_1, C_2, C_3) finally obtained were then assembled into three groups of similar I.V. as follows:

No.	g.	I.V.	No.	g.	I.V.
A	244	15·9	A_4	244	15·9
B	413	37·5	B_4	347	37·1
			D_1	66	40·6
C	368	57·4	C_1	118	56·3
			C_2	94	57·6
			C_3	87	57·6
			B_6	59	55·3
			D_2	10	52·8

(The weights actually obtained were slightly diminished owing to withdrawal of samples for I.V. determinations, etc., and have been "corrected" in the above description to allow for this; the total weight so withdrawn amounted to about 5 g., or 0·5 % of the original fat employed.)

The component fatty acids in each of the fractions A, B, C were determined by lead salt separation and ester-fractionation as in the case of the whole fat (Table I). The final percentage compositions (wt. and mol.) found for the acids from each fraction are shown in Table II.

Table II. *Component acids of the separated fractions (A, B, C) of ox depot fat*

	A		B		C	
Saturated:	% (wt.)	% (mol.)	% (wt.)	% (mol.)	% (wt.)	% (mol.)
Lauric	—	—	0·2	0·2	0·3	0·4
Myristic	1·3	1·5	2·1	2·6	2·5	3·0
Palmitic	44·0	46·5	29·8	31·7	25·0	26·5
Stearic	31·8	30·3	25·8	24·7	12·2	11·7
As arachidic	5·5	4·8	0·4	0·3	0·1	0·1
Unsaturated:						
Tetradecenoic	0·3	0·4	0·3	0·4	0·7	0·9
Hexadecenoic	1·1	1·1	1·5	1·6	2·6	2·7
Oleic	16·0	15·4	38·8	37·4	47·4	45·8
Octadecadienoic	—	—	1·1	1·1	9·0	8·8
As C_{20-22} unsaturated	—	—	—	—	0·2	0·1

It will be observed that, of the minor component acids, arachidic acid is the only one concentrated in fraction A, in which fully saturated and mono-unsaturated-disaturated glycerides predominate. Myristic, tetra- and hexadecenoic, and especially octadecadienoic and the unsaturated C_{20-22} acids, are found in the (mainly di-unsaturated) glycerides of fraction C, which are liquid at room temperature and most soluble in acetone.

Examination of fully saturated glycerides present in fractions A and B of the ox depot fat

The fully saturated glycerides were isolated, and their amounts determined, in each case by oxidation of the fats in acetone solution with powdered $KMnO_4$ by our usual procedure.

Fraction A: 80·2 g. gave 49·9 g. fully saturated glycerides (sap. equiv. 283·4, acid value 4·8, I.V. 0·2). *Fully saturated glycerides*: 59·0 % (wt.) or 60·0 % (mol.).

Fraction B: 200 g. gave 14·6 g. fully saturated glycerides (sap. equiv. 272·1, acid value 1·8, I.V. 0·4). *Fully saturated glycerides*: 7·1 % (wt.) or 7·4 % (mol.).

The component acids in each fraction of fully saturated glycerides were determined by ester-fractionation with the results given in Table III.

Table III. *Component acids of the fully saturated glycerides in fractions A and B*

	A		B	
	% (wt.)	% (mol.)	% (wt.)	% (mol.)
Myristic	5·4	6·3	7·5	8·6
Palmitic	49·6	51·5	63·0	64·2
Stearic	45·0	42·2	29·5	27·2

A portion of the fully saturated glycerides from the least soluble fraction A of the ox fat was systematically crystallized from anhydrous ether into five fractions. From the sap. equiv. of these fractions it was estimated that the material contained 3·0% (mol.) of tristearin and 19·6% (mol.) of tripalmitin (the latter figure is probably somewhat higher than the truth, because traces of acidic products of oxidation left in the fully saturated glycerides may have lowered the equiv. of the portion most soluble in ether).

Lack of material prevented similar crystallization of the fully saturated glycerides from fraction B, which however only amounted to 3% of the whole fat, and were taken as a mixture of tripalmitin and dipalmitostearin in the proportions demanded by the fatty acid composition.

Determination of tri-C_{18} glycerides in each fraction (A, B, C) of the ox depot fat

A portion (50 g.) of each fraction was completely hydrogenated and then submitted to systematic crystallization from anhydrous ether. From the sap. equiv. of the six or seven crystal fractions thus obtained the proportion of tristearin was determined [cf. Hilditch & Stainsby, 1935]. The molar percentage, of course, corresponds with the molar percentage of glycerides composed exclusively of C_{18} acids in the original fats before hydrogenation. The amounts of tristearin determined by this procedure in the hydrogenated portions A, B and C are shown in Table IV.

Table IV. *Tristearin content of hydrogenated fractions A, B and C of the ox depot fat*

Fraction	% (wt.)	% (mol.)
A	18·4	17·7
B	15·6	15·1
C	9·7	9·3

Discussion

The evaluation of the data recorded in the experimental part requires some explanation both as regards general interpretation and as regards the manner in which the various minor component acids in the fat are considered. The discussion is based throughout, it should first of all be noted, on the molar (not weight) proportions of the various components; this has the primary advantage that in any given case the same numerical figure denotes the proportion of a component either in the form of glyceride or of the fatty acid in question. The data for the molar proportions of glycerides in fractions A, B and C of the ox depot fat, and for the increments of component acids present in each fraction (from the percentage figures given in Table II), are shown in Table V.

Comparison of the last column in Table V with Table I shows satisfactory accordance in the percentages of the component acids as determined (i) in the whole fat and (ii) from summation of the component acids recorded for each of the three fractions A, B and C. As would be expected, the components present in very small proportions are more apparent in the data for the subdivided parts,

Table V. *Proportions (mol. %) of glycerides and of their component acids in fractions A, B and C*

	A	B	C	
Weight of fraction:	244	413	368	
I.V.	15·9	37·5	57·4	
Sap. equiv.	283·3	284·9	285·3	
Unsaponifiable %	—	0·06	0·2	
Glycerides % (wt.)	23·8	40·3	35·9	
Glycerides % (mol.)	23·9	40·3	35·8	
Component acids (increments % mol):				Whole fat
Lauric	—	0·1	0·15	0·25
Myristic	0·35	1·0	1·05	2·4
Palmitic	11·1	12·8	9·5	33·4
Stearic	7·2	10·0	4·2	21·4
As arachidic	1·15	0·1	0·05	1·3
Tetradecenoic	0·1	0·2	0·3	0·6
Hexadecenoic	0·3	0·6	1·0	1·9
Oleic	3·7	15·1	16·4	35·2
Octadecadienoic	—	0·4	3·1	3·5
C_{20-22} unsaturated	—	—	0·05	0·05

in one or other of which they have become concentrated. Indeed, arachidic and unsaturated acids of the C_{20} and C_{22} series (which are found only in the residual fractions of the distilled esters) only became detectable in the analyses of the fat after separation by crystallization.

The method of estimation of component glycerides employed in this work depends essentially on comparison of non-C_{18} and C_{18} acid components on the one hand, and of the total saturated and unsaturated acid components on the other. Complete detailed results can therefore only be obtained when the acids of the fat consist of one saturated non-C_{18} acid (e.g. palmitic), one saturated C_{18} acid (stearic) and unsaturated C_{18} acids (which, whether oleic or linoleic, etc., must be considered for the present purpose as one group; it is clearly impossible, by the use of methods involving hydrogenation, to distinguish between what were originally oleic or linoleic glycerides. To emphasize this, as in previous papers, the prefixes "oleic" or "oleo-" are employed when it is desired to indicate that all the C_{18} unsaturated components, and not merely oleic derivatives alone, are included).

In the seed fats investigated by this procedure, the acids present have been confined so far to palmitic, stearic, oleic and linoleic, with the exception of extremely small proportions of myristic or arachidic acids. In the case of ox depot fat (and of other animal fats which we are at present examining) the number of these minor components is increased and unfortunately includes, amongst others, about 2% of hexadecenoic acid (and traces of tetradecenoic acid), belonging simultaneously to the unsaturated group and to the group of non-C_{18} acids. In relation to the three major components (palmitic 33, stearic 21, oleic 35 ("oleic" 39), amounting together to 93% of the component acids) the quantity of any one of the minor components is almost insignificant; but it must be remembered that each mol. of a minor component will be associated almost invariably with 2 mol. of one or other of the three major components in a mixed triglyceride molecule, so that the total percentage of triglycerides involved is not of the order of 7%, but about 20%, of the whole fat. Whilst it is desirable to draw attention to this feature, it should equally be pointed out that the data presented in this paper are entirely valid from the point of view of the distribution of saturated non-C_{18} and C_{18} acids in the mixed glycerides of the depot fat,

and that the great predominance of palmitic acid in the former class ensures that the conclusions drawn later cannot be, in point of fact, very far from the actual state of affairs. It is perhaps not unreasonable to remark upon the fact that the presence of a number of minor component acids is the bugbear of detailed study of component glycerides in natural fats; in the rare absence of these, it is now possible to give an almost exact statement of the component glycerides present in a fat containing only three fatty acids.

For the purpose of the present discussion, myristic and lauric are included with palmitic glycerides, and arachidic with stearic glycerides, whilst the traces of unsaturated C_{20} and C_{22} acids are included in the "oleic" group. Hexadecenoic (and tetradecenoic) glycerides, which fall in the unsaturated non-C_{18} acid category, have been included also with palmitic glycerides, since on hydrogenation (for the tri-C_{18} glyceride determinations) they yield palmito- (or myristo-) glycerides. Accordingly, in terms of non-C_{18} ("palmitic"), stearic and "oleic" derivatives, the data in Table V, together with those for the fully saturated glycerides and contents of tri-C_{18} glycerides, may be transformed as shown in Table VI. In Table VI are also given the deduced proportions of palmitodi-C_{18}- and dipalmitomono-C_{18}-glycerides, and of mono- and di-unsaturated glycerides. Since the determination of the former and of tristearin, by crystallization of the hydrogenated fats from ether, is of a lower order of accuracy than the component acid analyses in Table II, the palmitodi-C_{18}- and dipalmitomono-C_{18}-glycerides have been calculated from the latter, after deduction of the proportion of tri-C_{18} acids present as tri-C_{18} glycerides. In fractions A and B, the fully saturated glycerides present are of course first dealt with, and the remaining acids accounted for in the various categories of mixed saturated-unsaturated glycerides.

Table VI. *Glyceride categories (% mol.) present in fractions A, B and C of ox depot fat*

	A		B		C	Whole fat
Glycerides	23·9		40·3		35·8	100·0
Component acids (increments):						
Palmitic	11·85		14·7		12·0	38·55
Stearic	8·35		10·1		4·25	22·7
"Oleic"	3·7		15·5		19·55	38·75
Component glycerides (increments):	Fully saturated	Mixed	Fully saturated	Mixed		
Tri-C_{18}	0·4	3·8	—	6·1	3·3	13·6
Palmitodi-C_{18}	5·8	0·8	—	24·9	29·0	60·5
Dipalmitomono-C_{18}	5·4	4·9	2·4	6·3	3·5	22·5
Tripalmitin	2·8	—	0·6	—	—	3·4
Mono-"oleo"-disaturated	—	8·0	—	28·1	12·9	49·0
Di-"oleo"-monosaturated	—	1·5	—	9·2	22·9	33·6

Consideration of the data in Table VI leads us to the statement of component glycerides in the fractions of the original fat, and consequently in the whole of the original fat, given in Table VII. These considerations are as follows.

Fraction A. The fully saturated components are derived from the estimated amounts of tripalmitin and tristearin, those of dipalmitostearin and palmitodistearin following from the component acid percentages in Table III. In the mixed saturated-unsaturated glycerides, the 4·9% of dipalmitomono-C_{18} glycerides must be "oleo"-dipalmitin. Of di-"oleo"-monosaturated glycerides, palmitodi-"oleins" are relatively soluble in acetone and may safely be considered absent from the sparingly soluble fraction A; the 1·5% of di-"oleo"-

glycerides is therefore credited as stearodi-"olein" and the remaining 2·3 % of tri-C_{18} glycerides as "oleo"-distearin, whilst the 0·8 % of palmitodi-C_{18} glycerides is taken as "oleo"-palmitostearin.

Fraction B. After reckoning the small amount of fully saturated glycerides (from the fatty acid analysis, Table III) as tripalmitin (0·6) and dipalmitostearin (2·4), and the remaining 6·3 % of dipalmitomono-C_{18} glycerides as "oleo"-dipalmitin, the 6·1 % of tri-C_{18} glycerides is assumed to be stearodi-"olein". (Some of the latter might be, perhaps, "oleo"-distearin, but since the amount of this relatively insoluble glyceride in the least soluble fraction A is only 2·3 % of the whole fat, it is certain that the proportion in fraction B will be definitely less than this.) Of the 9·2 % of di-"oleo"-glycerides, the remaining 3·1 % is then palmitodi-"olein", and the rest of the 28·1 % of mono-"oleo"-glycerides appears as "oleo"-palmitostearin.

Fraction C. From the proportions of saturated and unsaturated acids in this portion, its components lie between the following limits:

	% (of whole fat)
Mono-"oleo"-glycerides	12·9–24·4
Di-"oleo"-glycerides	22·9– 0
Tri-"oleins"	0–11·4

Since, however, the total amount of tri-C_{18} glycerides in C is only 3·3 % of the whole fat, there cannot at most be more than 3·3 % of tri-"olein" present (the corresponding figures in this case being 16·3 % of palmitodi-"olein", with 12·7 % of "oleo"-palmitostearin). But the presence of probably nearly 6 % of stearodi-"olein" in fraction B implies almost certainly that appreciable amounts of this glyceride will be present in the most soluble fraction C, and therefore, whilst the experimental data do not permit a final subdivision into tri-"olein" and stearodi-"olein", the probability is very great that nearly all the 3·3 % of tri-C_{18} glycerides in fraction C is, in fact, stearodi-"olein". The experimental figures, in fact, show almost conclusively that tri-"oleins", if not wholly absent, can only be present in quite insignificant amounts in the specimen of ox depot fat investigated.

The proportions of the remaining components of fraction C, once the nature of the tri-C_{18} unsaturated glycerides has been settled, follow as in the preceding cases of fractions A and B.

Table VII. *Probable component glycerides (% mol.) of the ox depot fat*

	A		B		C	Whole fat	
	Fully saturated	Mixed	Fully saturated	Mixed		Exact figure	(in round nos.)
Fully saturated glycerides (17·4 %):							
Tripalmitin	2·8	—	0·6	—	—	3·4	3
Dipalmitostearin	5·4	—	2·4	—	—	7·8	8
Palmitodistearin	5·8	—	—	—	—	5·8	6
Tristearin	0·4	—	—	—	—	0·4	†
Mono-"oleo"-disaturated glycerides (49·0 %):							
"Oleo"-dipalmitin	—	4·9	—	6·3	3·5	14·7	15
"Oleo"-palmitostearin	—	0·8	—	21·8	9·4	32·0	32
"Oleo"-distearin	—	2·3	—	—	—	2·3	2
Di-"oleo"-monosaturated glycerides (33·6 %):							
Palmitodi-"oleins"	—	—	—	3·1	19·6	22·7	23
Stearodi-"oleins"	—	1·5	—	6·1	3·3	10·9	11
Tri-"oleins"	—	—	—	—	*	*	*

* Traces of triolein, probably not exceeding 1 % of the fat, may be present.
† Tristearin is present to the extent of less than 1 % of the fat.

The ox depot fat investigated thus consists of about one-third of "oleo"-palmitostearins, and one-quarter of palmitodi-"oleins", with (in progressively diminishing proportions) "oleo"-dipalmitin, stearodi-"olein", dipalmitostearin and palmitodistearin. Very small amounts of tripalmitin, tristearin and (possibly) tri-"olein" are also present.

It is interesting to compare the detailed results obtainable by the procedure which has now been employed with those obtained by earlier methods. By simple but intensive crystallization of a sheep depot fat, Bömer *et al.* [1907; 1909] detected about 3% of tristearin with 4–5% of dipalmitostearin and some palmitodistearin. Banks & Hilditch [1931; 1932] were able to give limiting values for the mixed saturated-unsaturated glycerides by determining the total molar proportions of the fully saturated glycerides present. In the ox depot fat which has now been studied this procedure would have enabled us only to state the approximate components as fully-saturated 17·4%, mono-"oleo"-disaturated from 49 to 66%, di-"oleo"-monosaturated from 34 to 0% and tri-"oleins" from 0 to 17%. Determination of the tri-C_{18} glyceride content of the whole fat, coupled with consideration of the major component acids and the fully saturated glycerides, as applied by Hilditch & Stainsby [1935] to a pig depot fat, would have permitted a somewhat narrower range of the chief possible components: for example, 0–13·6% tri-"olein", 13·6–0% stearodi-"olein", 20–7% palmitodi-"olein", 34–47% "oleo"-palmitostearin, with 15% "oleo"-dipalmitin and 17·4% of fully saturated glycerides.

The procedure which has now been described, however, has demonstrated that tri-"oleins" are unlikely to be present in more than traces, and cannot in any case form more than 3% of the fat; whilst it has also indicated the presence of very small proportions (about 2%) of "oleo"-distearin. It therefore follows that the proportions of each of the component glycerides are defined by the approximate figures given in the final column of Table VII. (Even if the 3% of tri-C_{18} glycerides in fraction C were calculated alternatively as stearodi-"oleins" and as tri-"oleins", the possible limits of the component glycerides concerned would be narrowed to 0–3% tri-"olein", 11–8% stearodi-"olein", 23–20% palmitodi-"olein" and 32–35% "oleo"-palmitostearin.)

The possession of approximate figures for the chief component glyceride percentages permits some further discussion of the characteristic glyceride structure of ox depot fat in contrast to the "evenly distributed" type of glyceride structure encountered in the majority of natural fats. As mentioned earlier, the glycerides of several solid seed fats which conform closely to the "evenly-distributed" type have been investigated by the present method, and it has been found [Hilditch, 1938] that the observed proportions therein of the di-"oleo"-glycerides, and to a less extent the mono-"oleo"-glycerides, can be fairly closely reproduced by a simple calculation based upon the proportions of the component acids in the whole fat. If the chief component acids of the ox depot fat were assembled into a mixture of component glycerides on the lines of a typical seed fat (in which case there would be negligible proportions of fully saturated glycerides present), the application of the calculation referred to would suggest the probable presence of about 10% palmitodi-"olein", 6% stearodi-"olein", up to 62% of "oleo"-palmitostearin and 22% or more of "oleo"-dipalmitin—proportions which, naturally, differ widely from those observed in the foregoing analysis. If, on the other hand, allowance is made for the 17·4% of fully saturated glycerides known to be present, application of the numerical calculation to the component acids (28·0% palmitic, 15·8% stearic and 38·8% "oleic") of the mixed saturated-unsaturated glycerides would suggest the following composition

for the latter: 22% palmitodi-"olein", 12% stearodi-"olein", up to 35% of "oleo"-palmitostearin and 14% or more of "oleo"-dipalmitin. These figures are remarkably close to those actually observed (Table VII), but the implication of the coincidence, if it be more than a fortuitous one, is not at present altogether clear. Until the results of some contemplated parallel investigations of other animal depot and milk fats become available, it is therefore deemed desirable merely to draw attention to this feature without further comment, other than that an "even distribution" of the component acids of the mixed saturated-unsaturated glycerides seems not inconsistent with the hypothesis [Hilditch & Sleightholme, 1931; Banks & Hilditch, 1932; Hilditch, 1937, 1, 2] that the stearo-glycerides in the stearic-rich animal depot fats have resulted from saturation of preformed oleo-glycerides.

The hypothesis in question (with its extension to milk-fat glycerides) correlates the unusually large proportions of fully saturated glycerides present in many animal depot and milk fats with their possible formation by hydrogenation (depot fats) or other transformation (the lower saturated acids of milk-fats) of oleic groups already in combination as glycerides. The results of the present study may be briefly discussed in relation to this view. The ox depot fatty acids include, in molar proportions (Table VI), 38·5% palmitic (or, more strictly, non-C_{18} saturated acids), 22·7% stearic and 38·8% oleic (with octadecadienoic) acid, and of the glycerides 13·6% contain three C_{18}, 60·5% contain two C_{18}, and 22·5% contain only one C_{18} acyl group. The observed composition (Table VII) of these various groups was as follows:

	Tri-"oleo"	Di-"oleo"	Mono-"oleo"	Fully saturated
Tri-C_{18} glycerides	Nil or traces	10·9	2·3	0·4
Palmitodi-C_{18} glycerides	—	22·7	32·0	5·8
Dipalmitomono-C_{18} glycerides	—	—	14·7	7·8

If, in accordance with the above hypothesis, it be assumed that all these products have resulted from hydrogenation of preformed oleo-glycerides, their percentage proportions, at corresponding stages of the supposed hydrogenation, would be as below:

From	Unchanged %	1st stage %	2nd stage %	3rd stage %
Tri-"oleins"	Nil or traces	80	17	3
Palmitodi-"oleins"	37	53	10	—
Dipalmito-"oleins"	65	35	—	—

In catalytic hydrogenation of mixtures of mono-, di- and tri-oleo-glycerides in presence of nickel at 170–180° it has been shown [Hilditch & Jones, 1932; Bushell & Hilditch, 1937] that the process takes place in consecutive stages (i.e. only one oleo-group is saturated at a time) and that, whilst all three stages of hydrogenation proceed concurrently, the reduction of tri-oleo- to di-oleo-glycerides proceeds much more rapidly than that of di-oleo- to mono-oleo-glycerides, and the latter probably proceeds somewhat more rapidly than the production of saturated from mono-oleo-glycerides. Further, the relative concentrations of the different groups have an important influence on the amounts of the various semi-hydrogenated glycerides produced. The observed proportions of these products (illustrated in percentage form above) is that which would be expected if a biological hydrogenation process had followed the same course as addition of hydrogen by a nickel catalyst. Little or no tri-unsaturated glycerides are present, and the proportions of glycerides corresponding with the second and third stages of hydrogenation are much less than those of the initial

stages. Less of the original oleodipalmitin (as presupposed by the hypothesis) has been reduced than of the original palmitodiolein, but the amount of the latter present is nearly three times that of the former, and this is therefore also in keeping with the above-mentioned observations.

The observed presence of perhaps 2–3 % of tripalmitin in a fat which contains large proportions of stearic as well as palmitic acid is unusual. Tripalmitin has hitherto only been reported in cases in which the saturated acids of a natural fat consist almost wholly of palmitic acid (e.g. olive oil, palm oil, rabbit depot fat), and, when stearic and palmitic acids are both present in quantity (as in cacao butter and many other seed fats), any fully saturated components have been found to consist of mixed palmitostearins. The tripalmitin in ox depot fat is explicable, however, on the above "hydrogenation" hypothesis, since hexadecenoic acid is a minor component of ox depot fat, and saturation of any hexadeceno-palmitins present in the fat would, of course, yield tripalmitin.

Summary

A specimen of ox depot fat has been separated into fractions of varying degrees of solubility in acetone, and the component acids and glycerides present in each fraction have been investigated. The procedure leads to an approximate statement of the proportions of each of the major component mixed glycerides present in the fat. The approximate molar percentages of the more abundant glycerides are "oleo"-palmitostearin 32, palmitodi-"olein" 23, "oleo"-dipalmitin 15 and stearodi-"olein" 11 %; 17 % of fully-saturated glycerides are present, mainly dipalmitostearin and palmitodistearin, with very small amounts of tripalmitin and tristearin. Triolein is either absent, or only present in very small quantities. (Myristic, hexadecenoic, arachidic and other acids which are present to very small extents in the fat have to be included with palmitic acid in the analysis of the component glycerides.)

The proportions of the respective groups of tri-, di- and mono-"oleo"-glycerides, and of the fully-saturated glycerides, are fully consistent with the hypothesis that the stearo-glycerides in ox depot fat have resulted from saturation of preformed oleo-glycerides.

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CCXXXII. STANDARDIZATION OF THE RAT FOR BIO-ASSAY OF VITAMIN E[1]

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A survey of numerous studies concerned with the experimental production of vitamin E deficiency reveals a rather wide variation in the time necessary to induce sterility in rats of either sex. In male rats reared from weaning on vitamin E-deficient diets, the time of experimental feeding necessary to produce degenerative changes in the germinal epithelium may vary from 50 days or less [Mason, 1926; Ringsted, 1936] to 90–150 days [Evans & Burr, 1927; Kudrjaschov, 1930; Juhász-Schäffer, 1931]. It has also been the experience of many investigators that a variable proportion of female rats reared on vitamin E-deficient diets will complete one or more pregnancies before exhibiting a resorption-gestation typical of vitamin E depletion. This variable occurrence of initial fertility has proved particularly annoying and time-consuming in the preparation of rats for the bio-assay of vitamin E, and has necessitated the production of a proven resorption in all rats prior to their use in assay tests. Attempts to eliminate the occurrence of this phenomenon by the use of slightly rancid fat in the hopes of destroying possible traces of vitamin E in the experimental diets used have not proved particularly effective. Furthermore, such a procedure seems highly undesirable in view of the observations [Kudrjaschov, 1935] that products of a ketone nature formed in rancid fats are capable of preventing implantation in rats and may even cause resorption of the foetus if implantation is successful. Proper limitation of the vitamin E content of the diet supplied to mothers whose offspring are to be selected for vitamin E deficiency studies appears to be a much more successful and acceptable method for elimination of initial fertility [Ringsted, 1936], but its general applicability is limited by the care and expense involved in the preparation of stock diets containing a uniformly low content of vitamin E. More recently, Bacharach *et al.* [1937] report the occurrence of resorption-gestations in the first pregnancies of 96 out of 104 vitamin E-deficient rats examined prior to 1937, and in 100% of 316 rats tested since that time, but fail to mention the extent to which the lactating young had access to the maternal diet or the composition of the latter.

Although it has been rather generally accepted that the vitamin E reserves of young rats and the variable response of the latter to vitamin E depletion are significantly influenced by the vitamin E content of the maternal diet, no effort has been made to evaluate the mechanisms concerned. The present communication deals with an attempt to determine to what extent the vitamin E reserves of newly weaned rats are due to direct consumption of the maternal diet as the young adopt free-eating habits during the latter part of lactation, and to what

[1] This investigation was aided by a grant to Vanderbilt University School of Medicine from the Division of Medical Sciences of the Rockefeller Foundation, and by a special grant for technical assistance from the American Academy of Arts and Sciences.

extent they can be attributed to placental and mammary transfer. These studies have also demonstrated the reliability of a relatively simple procedure which completely eliminates the occurrence of initial fertility and greatly facilitates the preparation and standardization of rats to be used in the bio-assay of vitamin E, or for experimental studies relating to vitamin E deficiency.

Vitamin E storage in offspring given access to maternal diet

In order to determine the storage capacity for vitamin E in suckling rats, the offspring of mothers maintained on diets containing variable amounts of vitamin E were permitted access to the maternal diet during the period of lactation, after which they were reared upon a vitamin E-deficient diet (diet 68, Table II). The data summarized in Table I demonstrate conclusively that delayed

Table I. *Onset of sterility in vitamin E-deficient rats in relation to the vitamin E content of the diet available during lactation*

Group	No. of rats	Diet to which rats had access before vitamin E depletion was begun	Age when put on vitamin E-deficient diet days	Days on vitamin E-deficient diet before onset of sterility†		
				Min.	Max.	Average
A	24 ♀	Stock diet,* supplemented with a special suckling diet containing 20% ether-extracted wheat germ still containing appreciable amounts of vitamin E	21–30	160	324	240
B	10 ♂		25–29	150	200	180
C	13 ♀	Stock diet, supplemented with fresh lettuce 2–3 times weekly	21	90	218	151‡
D	18 ♂		21	95	160	130
E	10 ♀	Stock diet only	21	58	113	82§
F	5 ♂		21	80	100	90
G	178 ♀	Vitamin E content of maternal diet unrestricted and often excessive, but mother and offspring given vitamin E-deficient diet after 10th day of lactation	21	32	71	43·4‖
H	118 ♂		21	30	70	48·4

* The stock diet used in these studies was a commercial dog biscuit which affords a daily intake of from 2–3 times the minimal requirement of vitamin E for the rat.

† Onset of sterility in female rats was taken as the beginning of the first gestation period during which resorption occurred. In males this was estimated on the basis of histological examination of the testis and epididymis and can be regarded as only approximate.

‡ Two rats resorbed during first gestation, 5 completed one, 3 completed two and 3 completed three gestations before resorption occurred.

§ Resorption during first gestation occurred in 8 out of 10 rats.

‖ First litter fertility completely eliminated.

onset of sterility in rats of both sexes is roughly proportional to the vitamin E content of the maternal diet, when the young have access to the latter throughout the period of lactation. The particularly long depletion period in the rats of groups A and B is due partly to the higher content of vitamin E in the diet, as compared with the other groups, and partly to maintenance on the maternal diet beyond the 21st day of life (at which time the rats in our colony are as a matter of routine weighed, removed from the mother and placed upon the experimental diets). Attempts to hasten the onset of sterility in a number of animals in groups A to D by aeration of the lard at 100° for 10 hr. before its inclusion in the diet were unsuccessful. Even the unsupplemented stock diet conferred an appreciable storage in suckling rats permitted to consume it prior to the 21st day of life (groups E and F). However, when the offspring were denied access to the maternal diet and consumed only a vitamin E-deficient diet from the

assumption of free-eating habits, sterility supervened very early (groups G and H). The latter two groups represent a summary of results obtained in the experiments discussed in the following sections.

Vitamin E storage in offspring denied access to the maternal diet

Three groups of 13 rats each, containing an equal distribution of litter-mate sisters, were selected for breeding and reared upon the diets S, S+E and D+E, respectively (Table II). It was estimated that the S diet supplied approximately 2–3 times the minimal requirement of vitamin E for the rat, as determined by bio-assay of known quantities of the diet and measurements of the daily food consumption. It was estimated that the S+E diet supplied approximately

Table II. *Composition of diets*

Diet S		Diet S+E	
Stock diet (commercial dog biscuit)		Stock diet	80
		Fresh wheat germ	20
Diet D+E		**Diet 68**	
Casein*	22	Casein*	22
Cornstarch	54	Cornstarch	54
Lard	20	Lard	20
Salts†	4	Salts	4
Brewer's yeast	1 g. daily	To each 100 g. of this diet, 8 g. brewer's yeast and 2 g. cod liver oil were added at the time of feeding	
Cod liver oil	6 drops daily		
Wheat germ oil ‡	6 ,,		

* Finely granulated commercial casein found to be suitable for use in vitamin E deficient diets without further purification.

† Modification of the Osborne-Mendel salt mixture suggested by Wesson [1932].

‡ Kindly supplied by General Mills, Inc., Research Laboratories, Minneapolis, Minn.

15 times, and the D+E diet about 10 times, the minimal daily requirement of vitamin E. The rats were all maintained individually in wire cages with screen floors throughout the experiment. The reproductive performance was essentially the same in all three groups. Litters were limited to a maximum of six young. Six mothers whose lactation record was decidedly inferior were discarded. 90% of the 93 litters delivered by the remaining rats were successfully weaned, yielding 666 offspring with an average body weight of 42·5 g. at the 21st day of life. This is well above the minimum standard of 36 g. maintained in this laboratory for 21-day-old rats selected for studies relating to vitamin deficiency and reproductive performance. We have found that rats failing to meet this standard are decidedly inferior and frequently give very atypical results.

Throughout each lactation period the rats of the S and S+E groups were given diet 68 (Table II) and, to avoid too abrupt a dietary change, were also supplied with the regular diet until its removal on the 10th day of lactation. Those of the D+E group were deprived of the wheat germ oil supplement between the 10th and 21st days of lactation. By this means, the offspring of all three groups were denied any source of vitamin E except that obtained through placental transfer and through the mammary secretion.

It is of particular interest that the paralysis characteristic of the suckling young of mothers low in vitamin E [Evans & Burr, 1928] appeared in 20% of litters reared by mothers of the S group, but in none of the offspring of the S+E and D+E groups, indicating a significantly greater storage of vitamin E in the latter. It has since been found that this phenomenon rarely appears

when the vitamin E-deficient diet is substituted for the S diet at the 14th–15th days of lactation. We have failed to find in the paralysed rats, and in a large series of rats known to have been severely depleted of vitamin E from birth to adult life, the thyroid hypoplasia noted in such animals by Singer [1936] and Barrie [1937]; this confirms a recent report by Telford *et al.* [1938].

(*a*) *Placental and mammary transfer to female offspring.* Three groups of 92, 32 and 36 female rats selected respectively from the S, S+E and D+E mothers, were reared upon diet 68 and mated with normal males after they had attained body weights of approximately 150 g. Many began their pregnancies between the 30th and 40th days of experiment and, despite the occurrence of pseudo-pregnancy in 12% of rats, all were successfully inseminated after an average of 45 days (i.e. at 66 days of age). In all 160 rats the first gestation terminated in complete resorption. Graphic representation of the average body growth of each group throughout the gestation-resorption indicated that the resorptive process was more severe in the offspring from mothers on the S diet than in those from mothers on the S+E and D+E diets, which is in accord with the small protection afforded to the testes of male offspring from the latter two groups compared with those from mothers on the S diet, as discussed below. Although we have failed to note any significant difference in the responses of offspring from mothers on the three types of breeding diets when used for the bio-assay of vitamin E concentrates, the data upon which strictly comparative estimates can be made are limited to 10 rats of each group and are considered insufficient to warrant a more positive statement.

(*b*) *Placental and mammary transfer to male offspring.* Three groups of 35, 21 and 21 male rats selected respectively from the S, S+E and D+E mothers, averaging 44·6 g. in body weight at the 21st day, were reared upon diet 68. A total of 95 testes and corresponding epididymides obtained either by operative removal or at autopsy after various intervals of feeding were weighed, prepared for histological study and classified according to the predominance of one or more of the five stages into which the degenerative process has been arbitrarily divided [Mason, 1926]. Special reference should be made to the value of histological examination of the epididymis in confirming and augmenting conclusions drawn regarding the previous and current alterations in the germinal epithelium of the testis. The results of this analysis are summarized in Table III.

It will be noted that in the offspring of mothers fed on the S diet (series S, Table III) early evidence of testicular injury appeared after as little as 30 days of feeding. At this time (51 days of age) mature spermatozoa were usually making their first appearance in the ducts of the caput epididymis both in the vitamin E-deficient rats and in litter-mate brothers reared from weaning upon the stock diet. Although the rats of this series had been receiving restricted amounts of vitamin E from birth, and some had spontaneously recovered from mild degrees of paralysis apparent during the last few days of lactation, the prepubertal growth and maturation of the testes exhibited no significant inhibition. Further investigations are being made in regard to the latter question, and to the earliest period at which the irreparable nature of the testicular injury can be demonstrated. The onset of testicular injury was approximately 10 days earlier than that observed in the earlier studies of Mason [1926] and from 5 to 10 days earlier than that noted by Ringsted [1936] in rats reared by mothers on a breeding diet containing a low but adequate content of vitamin E and subsequently fed on a vitamin E-deficient diet. The testicular damage observed in testes obtained after the same interval of feeding, and in the different seminiferous tubules of any one testis, was unusually uniform, especially when compared with the

Table III. *Extent to which placental and mammary transfer of vitamin E affects the onset of testicular degeneration in male rats reared on a vitamin E-deficient diet*

No. days on exp. after weaning	Series S		Series S + E		Series D + E	
	No. of testes	Stage of degeneration	No. of testes	Stage of degeneration	No. of testes	Stage of degeneration
30	5	N?–1	—	—	—	—
40	3	N?–1	—	—	—	—
	9	1	1	N?	1	N?
	6	1–2	—	—	—	—
50	1	1–2	—	—	3	N
	5	2–3–4	7	N–N?	3	N?–1
	5	3–4–5	2	N?–1	2	1–2
60	—	—	6	N?–1	3	N?–1
	1	3–4–5	3	1–2	3	1–2
	4	4–5	3	2–3–4	3	2–3–4
70	—	—	2	2–3–4	2	3–4–5
	—	—	4	3–4–5	4	4–5
80	—	—	2	4–5	2	4–5
Total no. of testes	39		30		26	

picture presented in testes of rats maintained for prolonged periods on vitamin E-deficient diets before the onset of sterility.

In the offspring of mothers fed on the S + E and D + E diets (series S + E and D + E, Table III) testicular degeneration appeared approximately 20 days later than in the S series described above, demonstrating an appreciable placental and mammary transfer of vitamin E when the daily intake of the mother was increased to 10 to 15 times the minimal. Preliminary attempts to measure the extent of this transfer, by feeding different amounts of a carefully assayed concentrate of vitamin E to 21-day-old rats treated in the same manner as those of the S series, indicated that the smallest amount of vitamin E necessary to delay the onset of testicular degeneration 20 days was approximately twice the single curative dose necessary for preventing resorption in female rats.

(*c*) *Placental versus mammary transfer.* The results of another series of experiments, which indicate that the mammary gland is almost entirely responsible for the transfer of vitamin E to the offspring, warrant brief mention. Three rats were given approximately 200 times the minimal single curative dose of vitamin E[1] during their period of gestation. Immediately after delivery, and before the young had opportunity to suckle, the litters were exchanged with those of recently delivered mothers fed on the stock diet. One other rat was given approximately 400 times the minimal single curative dose distributed over a period extending from the 13th day of pregnancy to the 7th day of lactation. The 22 male and 18 female offspring without access to any dietary source of E during lactation were reared upon the vitamin E-deficient diet.

In six male offspring whose storage of vitamin E was limited to that received during intra-uterine life, the time of onset of testicular degeneration was essentially the same as observed in the rats of series S (Table III). In 12 males suckled by mothers whose storage of the excess vitamin E administered during gestation apparently permitted close to the maximum mammary transfer of vitamin E to the adopted young, the testicular injury appeared about 20 days later, as noted in the rats of series S + E and D + E (Table III). Prolonged feeding of the

[1] In the form of a concentrate prepared from wheat germ oil by molecular distillation; kindly supplied by General Mills, Inc., Research Laboratories, Minneapolis, Minnesota.

vitamin E concentrate during lactation was deemed inadvisable because of possible contamination of the young with traces of the vitamin from the body or food of the mother. In the one instance where excess of vitamin E was given during late gestation and the first week of lactation, the four male offspring exhibited an additional delay of about 10 days in the onset of testicular degeneration, when compared with the latter group. It is particularly noteworthy that the 18 female rats derived from the seven litters just discussed, successfully impregnated at an average age of 64 days, invariably resorbed during their first gestation.

It is hoped that studies now in progress using a greater excess of vitamin E and a much larger series of animals will afford a more adequate measure of the maximum and relative efficiencies of the placental and mammary barriers in the transfer of vitamin E. The preliminary observations reported above merely suggest that the vitamin E storage of the offspring of mothers fed on the S+E and D+E diets represents close to the maximum attainable through combined placental and mammary transfer, that this storage is almost entirely due to mammary transmission of the vitamin and that increasing the daily vitamin E intake of the pregnant and lactating rats to several hundred times the minimal requirement does not permit enough vitamin E to pass the placental and mammary barriers to prevent resorption during the first gestation in female offspring otherwise deprived of vitamin E.

Discussion

The results presented in this report are in close accord with other observations regarding the limited placental and mammary transfer of vitamin A [Dann, 1932; 1934; Baumann *et al.* 1934] and of vitamin D [McCollum *et al.* 1927] in the rat. Questions regarding storage capacity and excretion after administration of an excess of the fat-soluble vitamins, the "physiological" selectivity of the placental and mammary barriers and possible differences in storage capacity in the offspring during intra-uterine and postnatal life, are beyond the scope of the present discussion but offer interesting fields for further exploration.

The relative ineffectiveness of placental and mammary transfer of vitamin E in the rat, even when the maternal intake is exceedingly high, affords conclusive evidence that voluntary consumption of the stock diet by the young rat during the latter part of lactation is the primary factor responsible for the irregularity and delay frequently noted in the time of onset of sterility in vitamin E-deficient rats of both sexes. The complete elimination of initial fertility in female vitamin E-deficient rats through the simple expedient of substituting a vitamin E-deficient diet before the middle of lactation, with the production of offspring whose vitamin E reserves are very low from the time of birth and little influenced by minor variations in the vitamin E content of the maternal diet (which may be selected from the standpoint of cheapness and excellence for breeding purposes), should greatly facilitate the standardization of rats for the bio-assay of vitamin E and permit a much more accurate evaluation of assays conducted in different laboratories.

Furthermore, the established reliability of the first gestation period for assay tests (when properly controlled by the use of untreated litter-mate controls), which can be begun after an average of 45 days from the time of weaning, together with the economy of space and diet due to the smaller size of the test animals, are distinct advantages. The virginal state of the reproductive tract prior to the gestation used for the bio-assay test also appears appreciably to increase the

number of successful impregnations after positive matings [Bacharach *et al.* 1937] and should impart greater reliability to the assay procedure. We have obtained exceedingly uniform and satisfactory results in a series of more than 180 bio-assay tests conducted on rats prepared in the manner described.

In the present study, the male proved to be a more delicate indicator of vitamin E storage in the young rat than did the female. Certain problems relative to sex differences in storage capacity and vitamin requirement during pre-pubertal and post-pubertal life are being more thoroughly investigated in order better to evaluate the possible usefulness of the male rat for vitamin E assays.

Summary

The variable responses of young rats of both sexes to vitamin E depletion can be closely correlated with the vitamin E content of the maternal diet to which the young have access after the adoption of free-eating habits during the latter part of lactation. By substitution of a vitamin E-deficient diet for the maternal diet during the last half of lactation, to limit the source of vitamin E to that obtained through the placenta and mammary gland, the initial storage of the vitamin in newly weaned offspring is decidedly limited and but little influenced by the vitamin E content of the maternal diet.

When the maternal diet contains but several times the minimal requirement of vitamin E, the vitamin E reserves of the offspring are negligible and, when reared upon vitamin E-deficient diets, males exhibit the onset of testicular degeneration between the 50th and 60th days of life, coincidently with the first appearance of spermatozoa. Increasing the vitamin E content of the maternal diet to about 15–20 times the minimal delays the onset of testicular injury about 20 days, which represents about the maximum protection obtainable through combined placental and mammary transfer, as indicated by preliminary tests in which the maternal intake was increased several hundredfold. Most of the vitamin transfer is accomplished through the mammary secretion.

Female rats whose initial vitamin E storage is limited to that obtained through placental and mammary transfer invariably resorb during their first gestation period when reared on vitamin E-deficient diets, even when the maternal intake is increased several hundredfold. The value of these observations in the improvement and standardization of methods for the bio-assay of vitamin E is emphasized.

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CCXXXIII. THE EFFECT OF ADRENALINE ON EMBRYONIC CHICK GLYCOGEN *IN VITRO* AS COMPARED WITH ITS EFFECT *IN VIVO*[1]

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Much less appears to be known of the mode of action of adrenaline on the breakdown of liver glycogen than of its mode of action on muscle glycogen. In muscle it has been shown that adrenaline initiates a breakdown of glycogen to lactic acid not only in an isolated perfused limb but in pieces of excised muscle *in vitro* [Hegnauer & Cori, 1934]. Lately Nachmansohn *et al.* [1936; 1937] have shown that adrenaline will act even on a muscle "brei", increasing the rate of formation of lactic acid and causing an accumulation of hexosemonophosphate, which seems to indicate that adrenaline may be acting here as a specific accelerator of one of the enzymes taking part in the phosphorylated glycogen glycolysis system in muscle.

With respect to its action on liver glycogen it is generally accepted that adrenaline produces a rise of blood sugar by breaking down preformed liver glycogen to glucose. But attempts to demonstrate the effect of adrenaline as a direct accelerating action on the liver glycogenase enzyme, converting glycogen into glucose, have so far proved unsuccessful [quoted Hodgson, 1936].

The various possibilities of the mode of action of adrenaline on liver glycogen and blood sugar seem to be:

(1) A direct action: (*a*) on the glycogenase enzyme independent of the cell structure, though this would appear unlikely in view of the experiments quoted by Hodgson; or (*b*) on the intact living liver cell, either breaking down a spatial separation of enzyme and substrate in the cell as Lesser [1920] suggested, or increasing the permeability of the cell wall to glucose, thereby causing a shift in the equilibrium between glycogen and glucose in the cell.

(2) An indirect action not demonstrable in the isolated living cell inasmuch as the final picture in the whole animal might be the resultant of a number of different actions of adrenaline. For example, the inhibition of glucose utilization under the influence of adrenaline reported by Cori & Cori [1928; 1929] and Colwell & Bright [1930] might account in part for the rise of blood sugar, while the constriction of the intrahepatic vessels as shown to occur with adrenaline by Clark [1928], Emery & Griffith [1931] and McMichael [1932] might be the cause of relative hepatic anaerobiosis and thus of the breakdown of liver glycogen.

In the hope of being able to throw some light on the question as to whether the action of adrenaline on liver glycogen is direct or indirect, an investigation was made of the effects of adrenaline and anaerobiosis on the glycogen content of cultures of embryonic chick liver where the conditions are not complicated by

[1] This investigation has been carried out under a Studentship from Newnham College, Cambridge, and a Hospitality Fellowship from the Canadian Federation of University Women.

the presence of nerves and blood vessels. In the present paper the results of this investigation are described, together with a study of the glycogen content of the liver of the embryo chick during development and a comparison of the effects of adrenaline on its liver and muscle glycogen.

Methods

Tissue culture methods and histology. 11- to 16-day-old chick embryo liver was cultured in "hanging drop" cultures by the technique described by Doljansky [1929] with certain modifications. The tissue was cut into cubes about 1 μl. in size, which were planted on the surface of a firm plasma clot, containing 0·1 %, 1 % or 2 % sugar. The cultures were incubated at 38° with the liver tissue resting on the surface of the clot, and experiments performed on them after 4–6 days. By this time the cultures had grown out, partly by spreading and partly by cell division, into thin sheets of epithelial cells with a slight thickening at the centre, and their glycogen content could easily be seen by staining methods.

The glycogen content of the cultures was ascertained histologically in the following way: The cultures were stained first by the I_2 vapour method [Lewis, 1921] and the intensity and position of the glycogen staining noted; they were then fixed in 98 % alcohol and subsequently stained with Best's Carmine, haematoxylin or thionine being used as counter-stains. The agreement between the two different methods was satisfactory in all cases.

Reagents. All the adrenaline solutions used were made up from the pure crystalline solid (B.D.H. Epinephrine). Small quantities were weighed out and dissolved in an equivalent weight of *N* HCl, and diluted with Tyrode or Pannet and Compton solution immediately before using.

Glycogen determinations. Glycogen in embryo chick liver and muscle was determined by the method of Good *et al.* [1933], the sugar formed by hydrolysis being estimated by Somogyi's methods (Somogyi [1926]: for very small quantities of sugar, Somogyi [1937]). The liver and muscle samples were taken immediately after removing the embryo from the egg, and were dropped at once into weighed tubes of 30 % KOH.

Results

1. *Growth and glycogen content of liver cultures.* Liver cultures were grown from 6- to 16-day-old chick embryos and in all cases abundant glycogen was found in the cells, both in the centre of the explant and in the cells at the edge of the outgrowing epithelial sheet. The cultures usually remained healthy and retained their glycogen for 5 to 7 days after implantation without the medium being renewed in any way. Nordmann [1929], who made an extensive study of the growth of liver cells *in vitro*, claimed that all glycogen was broken down during the implantation and was only built up again by the liver cells at the end of the first 24 hr. But glycogen was seen histologically 6 hr. and 19 hr. after implantation, and chemical estimations by Heatley's [1933] micro-method of pieces of embryonic liver, cut up to the size of culture implants and left to stand in saline for 1 hr., showed glycogen contents of 0·315 to 2·17 % (dry wt.).

The percentage of sugar in the medium did not have much effect on the glycogen content of the cultures, but those grown in a 0·5 % or 1·0 % sugar medium remained healthy and retained their glycogen longer than those grown in a medium to which no sugar was added.

For experiments with adrenaline, cultures of liver from 11- to 15-day-old embryos grown in a 0·5 % or 1·0 % sugar medium were used 4–6 days after implantation.

2. *Effect of adrenaline on liver cultures.* Groups of cultures were chosen as much alike as possible in size and extent of growth. To one group a drop of adrenaline solution was added, while to the control group a drop of Tyrode solution was added, and the cultures incubated at 38°. After varying periods of incubation they were tested histologically for glycogen. Solutions of adrenaline in concentrations of from 1 : 100 to 1 : 1,000,000 were employed and the *p*H of the solution was always adjusted to the same as that of the Tyrode solution used for the controls. The cultures were stained for glycogen after 5, 10, 15, 20, 30 and 40 min. and 7, 11 and 16 hr. The adrenaline-treated cultures, even those left for 16 hr. with a drop of 1 : 100 adrenaline solution, showed no signs of necrosis or destruction of the epithelial sheet, and in no case was their glycogen content diminished in comparison with the controls.

As Blaschko *et al.* [1937] and others have shown that liver tissue is one of the most active in destroying adrenaline in the presence of O_2, it was possible that, in spite of the strong doses used, the adrenaline was being oxidized to inactive compounds before it could diffuse into the cells. The drop of adrenaline was therefore sucked off and renewed every ½ hr. for 3 hr., various concentrations of adrenaline being employed. There was no diminution of the glycogen content in the culture so treated, and it seemed unlikely that all the adrenaline added would have been oxidized before it could exert its effect.

In view of the possibility that in the absence of the pituitary the glycogenolytic action of adrenaline might be impared [Corkill *et al.* 1933; Cope & Marks, 1934; Bachman & Toby, 1936], an alkaline extract of ox anterior pituitary was prepared, according to the technique described by Cope & Marks [1934]. The fresh glands were ground up with acetone, the acetone removed by filtration and evaporation in air and the acetone-free tissue extracted with saline. This extract was added to cultures, both alone and together with adrenaline, in several doses for 40 min. No reduction in their glycogen contents, as compared with the controls, was seen histologically. In all about 50 cultures were treated with adrenaline under the various conditions described.

It seemed therefore that under the conditions of the experiments adrenaline had no direct effect on the glycogen of isolated liver cells.

There was, however, an important possibility to be investigated before these results could be taken as an indication that the glycogenolytic action of adrenaline was not a direct one. This possibility was that the young chick embryos from which these cultures were made might not respond to a dose of adrenaline *in vivo* with a fall of liver glycogen.

Vladimirov [1931] injected very large doses of adrenaline (1 ml. 1 : 100 in some cases) into the air sac of the hen's egg and obtained evidence that the blood sugar was raised in the 19- and 20-day, and occasionally in the 14- and 15-day embryo, though not in the embryo younger than 14 days. But the values for the blood sugar level of normal and adrenaline-treated embryos all varied enormously and the adrenaline was given usually in doses of 0·5–1 ml. The absorption of such quantities of fluid he reports earlier in the paper as causing a fall of blood sugar in the younger embryos, due to a dilution of the blood. This might easily conceal any slight rise of blood sugar that would be produced by the breakdown of liver glycogen in, say an 11-day embryo. In such an embryo the quantity of liver tissue, and therefore of liver glycogen, present is very small compared with the volume of blood circulating in the whole egg.

Estimations of the liver glycogen, rather than of the blood sugar, of such small embryos would therefore be more likely to reveal any glycogenolytic action which adrenaline might have.

Accordingly an investigation of the effect of adrenaline on the liver glycogen of chick embryos was undertaken, and at the same time its effect on muscle glycogen was studied.

3. *Effect of adrenaline on the glycogen of chick embryos.* As the literature contained no values for the liver glycogen content of the chick embryo earlier than the 14th day of incubation, estimations were first made of liver glycogen of normal embryos from the 8th to the 21st day (see Fig. 1). Appreciable quantities of glycogen are present from the 8th to the 12th day (0·2 to 0·9 %), followed by a sudden rise on the 13th or 14th day. The figures for each day of incubation show very wide variations, but the curve drawn through the average values shows the same rise up to the 19th day and subsequent fall at hatching as Vladimirov [1930] found.

The effect of adrenaline on the liver and muscle glycogen of 1-, 14- and 18-day-old embryos was then determined in the following way. A number of eggs, containing embryos of the same age, was divided into two groups, usually of about 6 to 10 eggs each. A small hole was made in the shell over the air sac, and with a hypodermic syringe 0·1 ml. 1:1000, and in a few cases 0·1 ml. 1:50,000 adrenaline was injected into the air sacs of one group, and 0·1 ml. Tyrode solution at the same *p*H was injected into the air sacs of the control group. The hole in the shell was sealed up carefully with paraffin wax and the eggs were incubated for 40 to 60 min. air sac uppermost. During this time all the injected solution was adsorbed and the surface of the air sac usually appeared white and dry. The embryos showed no ill effects after this treatment. They were removed from the eggs and their livers rapidly transferred to weighed tubes of 30 % KOH. Large strips of pectoral muscle were taken for the muscle glycogen determinations. In the case of the 18-day embryos samples from 1 or 2 chicks were used for each determination, in the case of 14-day embryos samples from 1 to 4 chicks, and in the case of 11-day embryos samples from 5 to 7 chicks. Altogether 174 chicks were used: 46 18-day, 67 14-day and 61 11-day.

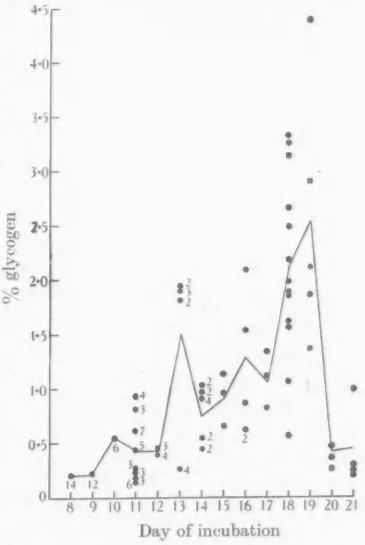

Fig. 1. Liver glycogen in chick embryos. Numbers denote the number of embryos used for a determination, if that number was greater than one.

As can be seen from Fig. 2, the glycogen contents of the livers of 11-, 14- and 18-day embryos treated with adrenaline were much reduced as compared with the saline-injected controls. It appeared therefore that adrenaline caused a mobilization of liver glycogen even in the youngest embryos from which liver cultures were made, and a lack of action *in vivo* could not therefore be the cause of the lack of action *in vitro*.

An investigation of the effect of adrenaline on embryonic muscle glycogen was of interest because of the findings of Needham & Nowinski [1937] that embryonic chick tissue was unable to glycolyse glycogen, being deficient, or partly so, in four of the essential enzyme components of phosphorylating glycolysis.

One of these enzymes was that responsible for the initial esterification of glycogen.

In Nachmansohn's [1937] experiments with frog muscle "brei" and adrenaline, and in Hegnauer & Cori's [1934] experiments with whole muscles placed in adrenaline solution, an accumulation of hexosemonophosphate was noted. It seems reasonable to suggest therefore that adrenaline might exert its accelerating effect at this initial step in lactic acid formation (*i.e.* the phosphorylation of glycogen). As slices of muscle from a 15-day chick embryo were unable to

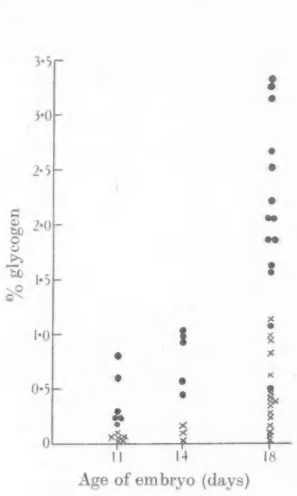

Fig. 2.

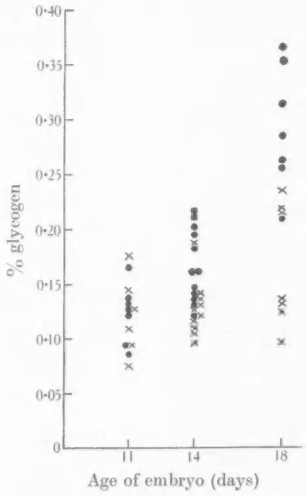

Fig. 3.

Fig. 2. Effect of adrenaline on embryonic chick liver glycogen.
• saline-injected embryos; × adrenaline-injected embryos.

Fig. 3. Effect of adrenaline on embryonic chick muscle glycogen.
• saline-injected embryos; × adrenaline-injected embryos.

glycolyse glycogen in Needham & Nowinski's experiments, it might be anticipated that adrenaline would fail to cause a breakdown of muscle glycogen before the 15th day. Fig. 3 shows that no significant difference between the muscle glycogen values of the adrenaline-treated and saline-treated 11- and 14-day embryos was obtained, whereas on the 18th day of development there was a quite well defined reduction in muscle glycogen under the influence of adrenaline. No experiments have been done to ascertain whether a "brei" of 18-day embryo muscle can produce lactic acid from glycogen, but it would appear from these results that the enzyme, or enzymes, on which adrenaline acts to cause a breakdown of muscle glycogen to lactic acid are present by the 18th day of development.

In the 11- to 14-day embryo the liver glycogen, but not the muscle glycogen, is reduced by adrenaline.

The failure of adrenaline to cause any breakdown of glycogen in the cultures of 11- to 14-day embryonic liver was therefore in contrast to its effects *in vivo*. There might of course be some condition, not fulfilled in the culture experiments,

which would enable a glycogenolytic effect of adrenaline to be demonstrated *in vitro*, but in view of the other possibilities of adrenaline's mode of action on liver glycogen it seemed of importance to study the effect of anaerobiosis on the glycogen of liver cultures.

4. *The effect of anaerobiosis on liver cultures.* Cultures were subjected to an anaerobic atmosphere in special flasks as illustrated in Fig. 4. The coverslip on which the culture was grown was placed on the plate-glass sheet *P*, and held in

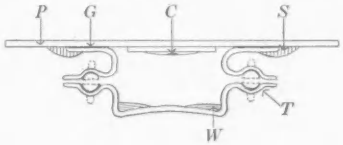

Fig. 4. Cross-section of apparatus employed for the subjection of cultures to an anaerobic atmosphere. *C* = culture on coverslip. *G* = ground glass surfaces. *P* = plate-glass sheet. *S* = sealing compound. *T* = glass taps. *W* = saline to keep atmosphere moist.

position with a little vaseline. A rubber grease was used between the ground-glass surfaces at *G*, and a sealing compound applied around the edges to complete the air-tight joint. The flasks were connected in series and a stream of N_2 and CO_2 mixture (95 % N_2 and 5 % CO_2) passed through them for 1 hr. The taps were then turned off and the flasks placed in the incubator. The culture could be observed microscopically at any stage of the procedure through the plate-glass sheet *P*.

Cultures were tested for glycogen after 1 to 6 hr. anaerobiosis at 38°. 10 hr. anaerobiosis were found to cause the death of all cultures so treated; the epithelial sheet appeared broken up and the individual cells rounded off. But cultures submitted to periods of 1–6 hr. anaerobiosis were found to remain healthy and to exhibit changes in their glycogen content.

Out of 45 cultures tested after 1–6 hr., 21 whose sister cultures all contained large quantities of glycogen were rendered completely glycogen-free.

Discussion

The experiments reported here on embryonic chick muscle glycogen gave results which are in agreement with the work done by Needham and his collaborators. However, the results of the experiments which show a breakdown of glycogen in cultures of 11- to 14-day embryonic liver under anaerobiosis and a reduction in liver glycogen in the intact embryo's liver after injection of adrenaline are somewhat difficult to reconcile with their findings. They used "breis" of whole 3- to 13-day-old chick embryos and found no glycolysis of added glycogen, although lactic acid was formed rapidly from added glucose. From the results with tissue cultures and intact embryos reported in this paper it appears, however, that there is present in the liver, though not always in muscle, an enzyme system capable of breaking down glycogen. In order to explain the difference between these results and those of Needham *et al.* it must be assumed either (*a*) that this enzyme system can attack glycogen which is actually in the liver cells of the embryo but not that added to the surrounding medium, or (*b*) that the glycogen is breaking down to a substance which is neither glucose nor lactic acid. The former suggestion would seem the more likely.

The difference between the effect of adrenaline on the liver glycogen of chick embryos *in vivo* and *in vitro* appears to indicate that the glycogenolytic action of adrenaline is not a direct one on the liver cells but is dependent on the presence

of factors obtained only in the whole body. Against this assumption, however, is the possibility that liver cells in a culture may be in a somewhat different metabolic state from those in the intact embryo. For example, cultures which have had their medium repeatedly renewed over a period of about 14 days cease to store any glycogen, owing probably to an abnormally high rate of proliferation induced in them, as Doljansky [1930] suggests.

Although the cultures used in the experiments here reported still retained their glycogen and were capable of utilizing it under anaerobiosis, it is possible that even during their 4 or 5 days of life in an artificial medium the cells had become altered in some way which would affect the action of adrenaline. For instance, a certain definite reduction potential of the various cell components might be necessary to enable adrenaline to exert its effect, and a shift in this potential, due to a change in environment, might thus render it inactive as a glycogenolytic factor.

Summary

1. Cultures of 11- to 14-day chick embryo liver, containing large quantities of glycogen easily observed by staining methods, were prepared. Adrenaline added to these cultures under a variety of conditions had no effect on their glycogen content.

2. Yet the chick embryo on the 11th, 14th and 18th days of incubation responded *in vivo* to doses of adrenaline with a well-marked fall of liver glycogen.

3. Although no reduction of muscle glycogen in the chick embryo under the influence of adrenaline was observed on the 11th and 14th days, there was a definite reduction on the 18th day of development. This result is in agreement with the results of Needham *et al.*, who found that the enzyme system in muscle, upon which adrenaline might exert its effect, was not present in the early stages of development.

4. Subjection of liver cultures to anaerobic conditions caused the complete breakdown of glycogen in a large number of cultures tested after 1–6 hr. anaerobiosis.

The author would like to express her gratitude to Prof. J. B. Collip for the privilege of working in his Department and to thank him and Prof. D. L. Thomson for the helpful criticism which they have given her.

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CCXXXIV. THE INFLUENCE OF COBALT ON PINE DISEASE IN SHEEP

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(*Received 30 August 1938*)

Pine disease in sheep is prevalent in certain parts of the Cheviot Hills on both the Scottish and English sides of the border, and a preliminary paper by Greig *et al.* [1938] described the nature of the disease and its occurrence. Evidence was submitted to show that it is an anaemia, chlorotic in type, and that it may be controlled by the administration of Fe compounds.

Nearly 200 pining sheep on various farms in the area have been treated with iron ammonium citrate or crude iron oxide during the past 3 years, a course of treatment lasting about 3 weeks being sufficient to bring about recovery in 95% of the cases. These results confirmed the conclusion reached earlier that these Fe compounds were of direct curative value in combating the anaemia and restoring the sheep to health.

Fe compounds are also effective in preventing the disease. Hill sheep in pining areas readily acquire a taste for iron oxide put into boxes with salt, and on many farms where this practice has been adopted the disease has been almost entirely abolished. Ewes which have received the crude iron oxide have been kept experimentally for 2 years on severe pining land without a change of pasture and have remained quite free from the disease.

As previously reported, however, it was observed that although Fe therapy proved effective it was not always followed by an increase in the blood haemoglobin and, further, the composition of soils and herbage from the area lent support to the view that Fe itself was not responsible for controlling the disease. In his study of enzootic marasmus in Australia, Underwood [1934] showed that the affected sheep had apparently no difficulty in absorbing ample quantities of Fe from food, for there was a higher concentration than normal of Fe in the liver, kidneys and spleen, but they lacked something necessary for the utilization of the Fe. It was shown that this disease, which resembles pine, could be cured by an extract, free from Fe, of the limonite commonly used by farmers and it was suggested that the disease was due, therefore, not to a deficiency of Fe in the food but to some trace element or elements associated with the Fe.

In the present investigation, attention was directed to Cu of which a trace was present in the iron ammonium citrate, but treatment of pining animals in the field with a dilute solution of $CuSO_4$ was ineffective. This result was not unexpected, because the analyses of soils and herbage from the area did not reveal any correlation between disease and Cu content.

Soils and herbage. At the beginning of 1935 samples of soil and herbage were taken from eight localities in the area in question and fresh samples were obtained from the same places in July. Altogether 73 samples of soil, to a depth of about 4 in., and 90 samples of herbage, including grass, heather and hay, were analysed, and a summary of the results is given in Table I.

In work of this nature it has been usual for investigators to determine the constitutents in a strong HCl extract of the soil, but it was felt that a closer approximation to the material available to plant and animal would be obtained by preparing a less acid extract; the analyses were, therefore, carried out on the filtrates obtained by extracting 10 g. soil with 250 ml. ammonium oxalate-oxalic acid solution containing 200 mg. equivalents of $HC_2O_4^-$ per l. and of *p*H 3·2–3·3. It was also considered desirable to express the results in terms of volume rather than of weight of soil, on account of the variation in soil type encountered, so the apparent densities of the soils were determined and the results calculated to a volume basis. Samples of the herbage were ignited at a dull red heat and the ash was extracted with dilute HCl.

Fe was determined by means of thiolacetic acid and Cu with sodium diethyldithiocarbamate, both colorimetrically, using a Lange photocell comparator. There is an indication that both soils and herbage from the non-pining land at D are better supplied with Fe and Cu than those from the pining land at D but they are no better supplied than those from the pining area A. Determinations of Mn, by the periodate method were no more successful in throwing light on the problem and it appeared to be unlikely that pine, if it were a deficiency disease, was controlled by any of the three elements Fe, Cu or Mn. Attention was then directed to cobalt.

Cobalt as a factor. Marston [1935] and Lines [1935] had reported favourable results from the administration of minute quantities of Co to sheep suffering from "coast disease" in Southern Australia. This was followed by a paper by Underwood & Filmer [1935] in which Co was reported to give positive results in cases of enzootic marasmus. Then it was discovered that "bush sickness" in New Zealand could be successfully controlled, both in South Island and in North Island, by the use of Co; this work has been summarized by Hopkirk [1938]. In view of these results, an analysis of the crude oxide of iron used in the experiments in the Cheviot region was made, and this showed that Co was present to the extent of over 50 parts per million. It seemed probable, therefore, that the good results obtained from Fe compounds in the control of pine disease might similarly be due to the presence of Co.

Soils. Several publications on the relationship between sheep disease and Co have appeared in the last 2 years, and in a recent paper Kidson [1938], summarizing the available data on the Co content of soils, shows that figures varying between 0·0 and over 300 p.p.m. have been obtained. These are for concentrated HCl extracts and are therefore not strictly comparable with the results in Table I, but it would appear that the Border soils are generally very low in Co. The method of analysis [Kidson *et al.* 1936] is based upon the depth of orange colour formed by the reaction of Co with nitroso-*R*-salt and is subject to considerable error when the amount of Co in solution is of the order 0·001 mg., but it will be observed that the soils from Farm C and Lower land D (non-pining) contain more Co per unit volume than the others.

Herbage. The analyses of pastures in New Zealand [Askew & Dickson, 1937; Askew & Maunsell, 1937] have shown that the dry matter of unhealthy pastures usually contains less than 0·07 p.p.m. Co, whereas, for good quality pastures, the Co content is generally greater than 0·07 p.p.m. Paterson [1937], on the other hand, has recently suggested that 0·2 p.p.m. is insufficient and that 0·45 p.p.m. is sufficient in the pastures of Devon. The values given in Table I for the herbage lie between 0·20 p.p.m. and quantities less than 0·05 p.p.m. and those samples from C and D are rather better than the others. It would be quite impossible at present to draw a line showing the limiting value and there

Table I. *Average figures for minor elements in soils and herbage**

	Soil							
	Spring				Summer			
Place	Fe	Cu	Mn	Co	Fe	Cu	Mn	Co
A. Pining farm, hill	92	29	80	1·2	193	48	147	—
Pining farm, field *a*	218	36	21	1·8	201	49	23	1·7
Pining farm, field *b*	222	48	47	2·0	145	32	34	2·0
B. Pining farm, peat	48	18	0·3	1·5	41	11	0·5	0·8
C. Non-pining farm, peat	141	36	2	2·8	68	16	0·8	0·8
Non-pining farm, mineral soil	248	34	64	5·4	519	59	108	3·7
D. Upper land (pining)	62	20	4	1·7	70	23	7	1·6
Lower land (non-pining)	229	36	46	3·0	298	43	20	3·7

	Herbage							
	Spring				Summer			
	Fe	Cu	Mn	Co	Fe	Cu	Mn	Co
A. Pining farm, hill	18	49	116	0·15	7	42	69	0·10
Pining farm, field *a*	—	—	—	—	8	44	91	
Pining farm, field *b*	—	—	—	—	—	—	—	—
B. Pining farm, peat	12	24	54	Tr.	13	15	90	Tr.
C. Non-pining farm, peat	15	23	45	Tr.	13	16	56	Tr.
Non-pining farm, mineral soil	21	39	72	0·17	14	41	63	0·11
D. Upper land (pining)	20	54	33	0·17	6	35	51	0·07
Lower land (non-pining)	33	50	42	0·20	8	38	69	0·12

* Soils: Cu and Co p.p.m. by volume; Fe and Mn p./100,000 by volume.
Herbage: Cu and Co p.p.m. by weight; Fe and Mn p./100,000 by weight.
Tr. signifies less than 0·05 p.p.m.

is no definite relationship between soils and herbage. It is interesting to observe that if, as indicated by the New Zealand experiments, from 0·1 to 1·0 mg. Co per week is necessary to maintain health, a foodstuff containing only 0·1 p.p.m. cobalt (i.e. 1 mg. Co in 22 lb.) might be regarded as being adequately supplied with this minor element. Attention is directed to the fact, however, that there is a tendency, as shown by Askew & Maunsell [1937], for the Co content to fall during the growing season, so that individual results may be liable to misinterpretation. It was obviously necessary to test whether Co was the limiting factor in the health of the sheep by administering a supplement of Co to affected animals.

Preliminary field tests with cobalt. In May 1937 four pining Cheviot ewes were confined on a piece of pining hill land which had been found by previous tests to accentuate the trouble in a very marked degree. The ewes were given doses of 3 mg. Co per head in the form of $CoCl_2$ (Analar) in distilled water. This dosage was given daily for 1 week, and thereafter every second day, a total of 12 doses being administered to each sheep. Within 10 days an improvement in the appearance and vigour of the sheep was noted, and after 17 days recovery was regarded as being complete. All four ewes nursed lambs throughout the test and these also improved along with their mothers. The ewes increased, on the average, by 7·87 lb. live weight per head in 3 weeks from the date of commencement of the test. They were thereafter returned to their hill grazings and continued to make good progress throughout the summer.

In June 1937 a further three pining ewes were treated on similar lines and these also made a rapid recovery. They increased in live weight, on the average, by

8·56 lb. per head in 3 weeks. In 6 weeks the increase amounted to 15·4 lb. per head.

As 3 mg. Co per day had proved effective in the above instances it was decided to try smaller doses. A test was made on a pining farm in a different part of the Cheviot area during August, 1937. Five pining hoggs and one pining ewe were treated with 1 mg. Co per head per day. Within 3 weeks they had lost all symptoms of the disease and were considered to be effectively cured.

As these preliminary tests had proved successful it was arranged to carry out a properly controlled experiment.

Controlled experiments with cobalt. Twenty Cheviot weaned lambs, or hoggs, which were healthy and free from pining, were divided into two groups on 16 August 1937, and enclosed on a section of pining hill land at an elevation of 800 ft. Both groups ran together and received no feeding except grass. An experiment on the same land during the previous season had shown an incidence of pining to the extent of 73 % within 2 months. The conditions were, therefore, favourable to the manifestation of the disease. The treatment given was as follows:

Group 1. 10 hoggs; 1 mg. Co per head per day for 14 days.
Group 2. 10 hoggs; no treatment.

Co was given in the form of $CoCl_2$ (Analar) dissolved in distilled water. The animals were dosed individually, the doses being spread over a period of 3 weeks, this being equivalent to the length of time normally occupied by a change off pining land.

The treated hoggs made good progress and remained entirely healthy. The untreated hoggs, on the other hand, soon began to develop symptoms of the disease. While all were healthy on 16 August, 8 out of 10 were pining by 11 September, and by the end of September only one hogg remained in a healthy state. The average live weights of the hoggs were as follows:

	16 August lb.	5 October lb.	Increase lb.
Treated group	38·35	46·25	7·9
Untreated group	36·4	39	2·6

The treated group gained 7·9 lb. live weight per head over the period, while the untreated group only gained 2·6 lb. There was an increase in the colour of the conjunctiva of the treated group, as ascertained by comparisons with a Tallqvist haemoglobin scale, while in the case of the untreated group, there was a decrease in the colour which was found on the average to amount to 9 %. It was clearly demonstrated by this test that the administration of 1 mg. Co per day for 14 days had entirely prevented the onset of pining.

The procedure next followed was to dose the untreated group, all of which, except one, were now affected with the disease. It was decided to attempt to cure the disease by doses of 0·5 mg. Co per head per day. Accordingly, 14 doses were given at this rate, spread over 3 weeks, commencing on 5 October. By 21 October quite a marked improvement was noted in the majority of the group, and within 1 month they had all recovered except two which had been particularly badly affected. These were given five additional doses of 3 mg. Co per day, after which recovery was rapid.

All the hoggs continued to run on the same section of pining land till 9 December and received no hand feeding of any description. Owing to heavy snow and frost thereafter, however, it was necessary to feed hay, the produce of

pining land. This treatment continued till 15 February, after which concentrates were fed and the experiment was terminated.

The original pining group increased in live weight by 3·2 lb. per head between 5 October and 9 December and, considering the severe conditions at this altitude of 800 ft., this afforded good proof of their recovered vitality.

While this experiment was in progress an outbreak of pining occurred among ewes and hoggs on the same hill farm, 30 animals being affected. Treatment was administered by giving for 3 weeks 1 mg. Co per day mixed with salt, together with a little concentrated food. As some of the sheep would not eat this mixture, they were given doses in liquid form. Recovery took place in all cases.

Discussion of results

The influence of Co in animal nutrition has been a subject of investigation by several workers. Waltner & Waltner [1929] found that the addition of Co to the diet of rats produced a marked increase in red blood cells. A similar result was noted in dogs by Mascherpa [1930], and other workers have confirmed these results. Beard & Myers [1929] found that Co was a useful supplement in curing anaemia in rats produced by feeding them with cow's milk, but Cunningham [1931] doubted whether a true anaemia was developed in rats fed on this diet.

Dixon [1937] showed that a weekly drench containing 0·8 mg. Co gave a good response in the control of bush sickness, and the effect was augmented by inclusion of 0·18 mg. Ni later in the treatment, when presumably the "primary deficiency of cobalt was satisfied". Filmer & Underwood [1937] found that 0·1 mg. Co per day was effective in controlling enzootic marasmus in Western Australia, whilst Marston *et al.* [1938] have shown how Cu, although useless alone, has a marked effect when administered along with Co in the treatment of coast disease of Southern Australia.

In the present investigation it has been shown by analysis of herbage that the amount of Co available to sheep on pining land is very small. The administration of 14 mg. of Co per sheep, however, entirely prevented the disease for 6 months on severe pining land. The consumption of Co over the whole of this period was less than 0·1 mg. per day.

Regarding therapeutic results from Co, a cure was repeatedly effected in the experiments described above by giving 14 doses of 1 mg. each, spread over a period of 3 weeks and the cure remained effective for many months. Attempts to deal with the disease with 0·5 mg. doses were only successful where pining was in its early stages.

It may be concluded from these experiments, therefore, that for preventive and curative purposes 1 mg. doses of Co are necessary to ensure reliable results in the treatment of pine disease in sheep, and that a minimum of 14 doses is desirable. The cost of the material is only about one penny for 30 sheep. These results have been obtained by administering the Co in solution to the animals individually, but the most practicable method of treating hill sheep is to allow the stock access to a mineral mixture containing an adequate amount of $CoCl_2$. The whole of the stock on a pining farm were treated in this manner, during the winter and spring of 1937–38, with complete success. The application of $CoCl_2$ mixed with fertiliser or sand to pining land merits consideration, however, in some of the more accessible places.

SUMMARY

1. Pine disease in sheep in the Cheviot region of Scotland is a nutritional anaemia which can be cured and prevented by the administration of cobalt in the form of cobalt chloride.

2. The disease is not due to a deficiency of Fe or Cu or Mn.

3. The beneficial results previously obtained from the feeding of Fe compounds may be attributed to the presence of Co in the Fe compounds used.

4. The administration of 1 mg. Co per day for 14 days is sufficient to prevent the disease on severe pining land for a period of 6 months. A similar quantity is effective as a cure.

The authors are grateful to Dr Comrie and Mr Sellar for assistance in the analyses of the samples of soil and herbage.

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CCXXXV. STUDIES ON DIFFUSING FACTORS. ACTIVE PREPARATIONS FROM MAMMALIAN TESTICLE AND THEIR BIOLOGICAL ASSAY

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Aqueous extracts of mammalian testicles contain a factor which dramatically increases the permeability of the skin to injected fluids and particles [McClean, 1930; 1931; Hoffman and Duran-Reynals, 1931]. An intracutaneous injection of saline, serum or an extract of any organ other than testicle produces a well-marked bleb, the margins of which remain quite distinct for 20–30 min. A similar injection of testicular extract, on the contrary, immediately diffuses into the dermis so that after 30–60 sec. it is difficult to identify the site of injection.

It can be shown that this diffusing factor is active in very low concentrations in the following way. Ascending dilutions of the extract are mixed with a constant amount of indian ink or a solution of diphtheria toxin, and the mixture is injected intracutaneously into the shaved back of a rabbit. Control injections of indian ink or toxin mixed with saline are also made. It is then observed that the area over which the particles of indian ink spread, or the size of the cutaneous lesions due to diphtheria toxin, is increased by high dilutions of testicular extract.

The factor responsible for the effect is associated with the germinal epithelium of the testicle, and is also found in extracts of spermatozoa [McClean, 1931]. Subsequent work has revealed that factors with similar diffusing properties can be obtained from the most diverse sources; e.g. from filtrates of invasive strains of staphylococcus and streptococcus [Duran-Reynals, 1933], from organisms of the gas-gangrene group and virulent pneumococci [McClean, 1936], from extracts of malignant tissues [Boyland & McClean, 1935], from snake and spider venoms [Duran-Reynals, 1936] and from leech extracts [Claude, 1937]. It appears therefore that these diffusing factors may have considerable physiological and pathological importance, and their chemical isolation is desirable.

A method of purification of the diffusing factor from bull's testicles has been described by Morgan & McClean [1932]. They estimated the activities of different fractions by determining the highest dilution which would produce an increase of at least 20% in the area of the cutaneous lesion produced by a standard dose of diphtheria toxin. This method is not sufficiently reliable for quantitative assay of diffusing factors for the following reasons: the skins of the experimental animals vary in their susceptibility to the toxin and to the diffusing factor; no standard diffusing preparation is used against which samples of unknown potency can be compared in each animal; the time of evolution and the type of the toxin lesion varies in different animals, and therefore the area of the lesions is not always clearly circumscribed and cannot always be determined at the same interval after injection; it is quite impossible to distinguish between successive tenfold dilutions of a given solution, and it is doubtful whether hundredfold dilutions can be consistently differentiated.

For further purification of the diffusing factor a more accurate method of titration is required. The use of indian ink as an indicator for the quantitative estimation of purified samples has been criticised by Favilli & McClean [1934]. They found that particles of indian ink adsorb the partially purified diffusing factor and that the amount adsorbed varies in different preparations. Furthermore, when indian ink is used it usually remains accumulated at the site of injection, and only a small amount spreads round it forming a halo of faintly stained tissue. The boundaries of this halo are not sharp enough to allow accurate measurements. The ideal indicator would be a soluble colouring matter which is not absorbed by the skin tissues, which spreads uniformly but not excessively, and is not removed too quickly. Such properties are possessed in large measure by haemoglobin. During the first few hours following an intracutaneous injection of a solution of a foreign haemoglobin the deeply pigmented spot at the site of injection is surrounded by a halo of less pigmented tissue. The outer boundaries of this halo are sharp and can be accurately measured. After 2–3 hr. the haemoglobin is evenly distributed over the whole area, which is approximately elliptical in shape. The stained areas produced by intracutaneous injection into different rabbits of a fixed amount of haemoglobin solution containing a given concentration of diffusing factor are relatively constant in size. Thus if the same titration is repeated in several animals the average of the results will not be influenced by extreme values obtained in a particular rabbit.

The haemoglobin does not disappear from the skin for at least 30 hr. The rate of increase of the coloured areas during this time can be determined. It has been found that the area coloured by haemoglobin increases rapidly during the first hour, the rate of increase being proportional to the concentration of diffusing factor. After 2–3 hr. it becomes independent of concentration (Fig. 1). The area of a coloured patch produced by a certain concentration of diffusing factor is always larger than that corresponding to an injection of a hundredfold dilution. The results are reproducible if similar injections are made in different places on the back of the same rabbit. If the results obtained in three rabbits are averaged it is possible to distinguish between tenfold dilutions. The experimental error is too large to allow closer titrations (Fig. 2).

The following standard procedure was followed throughout this work.

To compare the activities of two preparations of diffusing factor a series of tenfold dilutions of each is prepared. An isotonic solution of haemoglobin is used as a diluent. Intracutaneous injections of each concentration of one preparation are made in one side of the shaved back of each of three rabbits. In the other flank injections of the corresponding dilutions of the second preparation are made. Dilutions containing the same concentration should give coloured patches of similar area and moreover their rates of spread should be the same (Fig. 3).

For testing solutions of unknown activity, comparison is made with a standard stable preparation of diffusing factor, stored in the form of a water-soluble powder. The diffusing activity of this standard has been determined by establishing the smallest concentration at which it produces a definite increase in the rate of diffusion of haemoglobin and in the area of tissue over which it spreads. Since the susceptibility of the skin of individual rabbits to the diffusing factor may vary somewhat, determinations were made on five animals and the mean of the results was taken as a measure of the diffusing potency of this preparation (Fig. 4).

A considerable concentration of the diffusing factor present in aqueous extracts of bull testicles has been achieved by Morgan & McClean [1932]. They

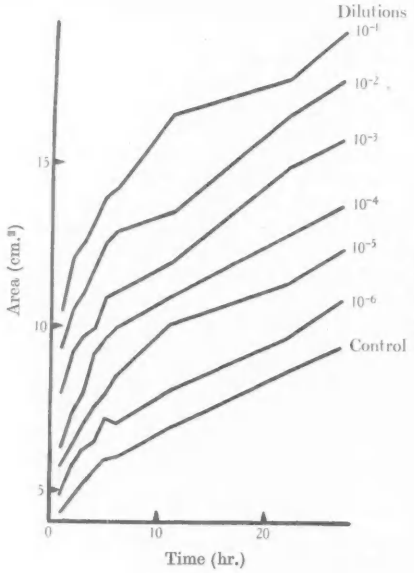

Fig. 1.

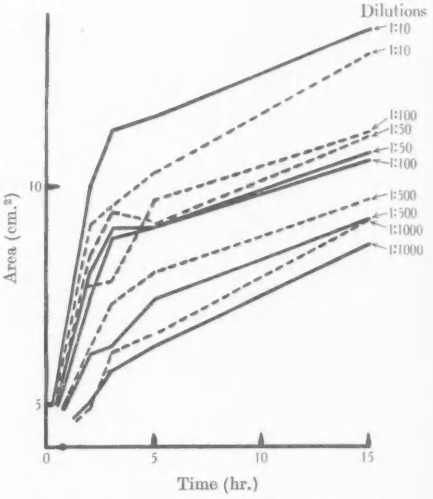

Fig. 2. —— Right flank. --- Left flank.

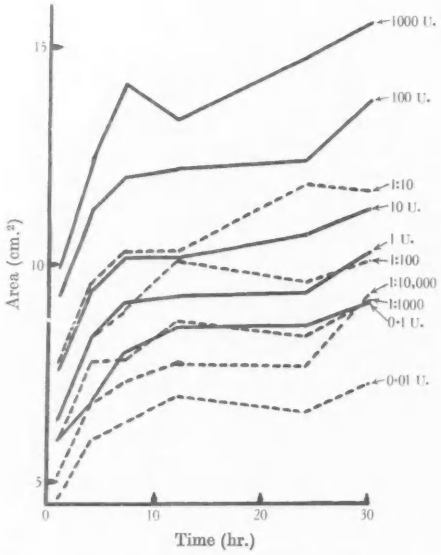

Fig. 3. —— Standard. --- Unknown.

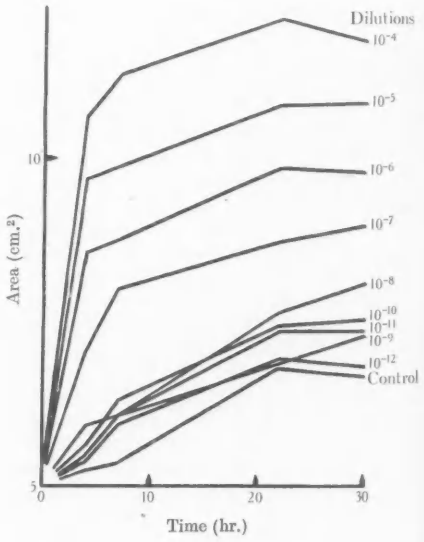

Fig. 4.

found that most of the protein could be removed from such extracts by precipitation with neutral lead acetate. From the filtrate the diffusing factor, together with $Pb(OH)_2$, was precipitated on making alkaline. The active material could also be precipitated with basic lead acetate. Claude & Duran-Reynals [1937] have described another method of concentration. This method involves treatment of crude testicular extract with acetone and fractionation of the redissolved acetone precipitate with $(NH_4)SO_4$. The active material precipitates with fully saturated but not with half saturated $(NH_4)_2SO_4$. Preparations obtained in this way obviously contain large amounts of protein, and, since Claude & Duran-Reynals did not employ a quantitative method of assaying biological activity, it is difficult to form any idea of the degree of purification attained by them.

Preparations containing relatively little protein have now been obtained by a method similar to that used by Morgan & McClean, save that the basic lead precipitate was decomposed with H_2S and the active material precipitated from the Pb-free solution with acetone. In agreement with the results of earlier workers, the diffusing factor was found to be non-dialysable (cellophane membranes) and insoluble in non-aqueous solvents with the exception of glacial acetic acid. Readily soluble in 50% alcohol, it is insoluble at concentrations of alcohol greater than 70–75%. The failure to dialyse must be regarded as a well established fact since as little as 0·01 μg. of active material per ml. can be detected biologically. This might indicate that the substance causing increase in dermal permeability has a high mol. wt.

The results of ultracentrifugal sedimentation, on the other hand, seem to indicate a relatively low mol. wt. Dr A. S. McFarlane (of this Institute), to whom my thanks are due, carried out an experiment in which a 2% solution (pH = 6·2) of an active material (1000 units per ml.) was centrifuged. The density of this preparation was measured pyknometrically at 25° and found to be 1·40. Very little sedimentation was observed after 3 hr. in a field 300,000 times gravity. The degree of sedimentation observed was such that the mean mol. wt. could not be greater than 10,000. The boundary was not sharp.

Some of the effects of purified preparations of diffusing factor on compressed unimolecular films were studied by Dr J. Schulman (Cambridge) to whom I also wish to express my thanks. The surface potential of a gliadin monolayer was substantially changed on adding the preparation. The alteration observed was of nearly the same order as that produced by a similar concentration of saponin. Unlike saponin, however, the tested preparation had no effect on the surface potential of a cholesterol monolayer.

The stability of purified concentrates of diffusing factor in solution at different pH values has been studied. At pH 3 complete loss of activity occurred after 24 hr. at room temperature. No loss of activity occurred at pH 4, 5, 6, 8 or 9. At pH 11·5 noticeable destruction (about 90%) was observed, while activity was completely destroyed after 24 hr. at pH < 3 or $> 11{\cdot}5$. Dilute solutions (0·1%, pH 7) of the purest preparations available lost their activity rather easily on heating. At temperatures of 60° and over, more than 90% of the activity was lost in 5 min. At 37° the solutions were much more stable, more than 90% of the activity being retained after 24 hr.

These results appear to be at variance with those of Claude & Duran-Reynals [1937] and Aylward [1937] who found some of their preparations to be relatively stable. The fact that these workers did not use a quantitative assay method may explain their results, since large changes in the concentration of diffusing factor solutions cause relatively little change in the area over which haemoglobin spreads in experimental animals.

The purest preparations of diffusing factor so far obtained are obviously mixtures of different substances. The only pure compound so far isolated from them is *meso*-inositol. This substance crystallises on addition of alcohol to dilute aqueous solutions of purified preparations. It can also be isolated from them by vacuum sublimation. Neither inositol nor its phosphate, phytin, show any diffusing properties in biological tests.

Experimental

Isotonic solution of haemoglobin. A measured amount of defibrinated sheep's blood is centrifuged and the serum decanted off. After washing with isotonic saline the erythrocytes are lysed with distilled water and made up to the original volume of blood. To render this solution isotonic 0·1 vol. of 8·5 % NaCl is added. A solution prepared in this way is used throughout as a diluent for preparing dilutions of the material to be tested.

Injections. Young male rabbits with unpigmented skin are used throughout. Rabbits with coloured fur are preferable. The back and flanks of the animal are shaved with electric clippers.

The rabbits are gripped as for measuring the coloured areas (see below). A line is drawn along the backbone of the animal and each flank divided into a number of squares corresponding to the number of injections to be made. The length of back generally available is 15–20 cm. and the areas coloured by the haemoglobin have a diameter of 2–5 cm. Therefore only five injections can be made on each flank. In order to use most of the available space the injections on each flank are made alternately at 1 and 2 cm. from the backbone. Only one row of injections can be made on each side of the rabbit.

The material is injected intracutaneously and deeply coloured weals result. When high concentrations of diffusing factor are injected the weals flatten almost immediately and the haemoglobin spreads rapidly over a large area of skin. 0·3 ml. is injected into each site; smaller amounts give areas difficult to measure accurately, and when larger volumes are injected, the injected fluid is apt to leak back through the point of injection.

Measurement of the coloured areas. The rabbits are gripped by the ears and the upper part of the hind legs and held on the knees of a seated assistant. Unnecessary strain of the animal's body is to be avoided. The longest (D) and shortest (d) axes of every coloured area are measured ($\pm$ 1 mm.) with calipers. The area (S) is calculated assuming that the coloured patches are regular ellipses $\left(S=\pi\frac{D.d}{4}\right)$. Variations due to the position in which the rabbits are held can be partially avoided by releasing the rabbit and repeating the reading after a short time. The average of three consecutive readings is taken as the area of the stain.

Less than 5 min. are required to measure the areas on each rabbit. During the first hour following injection the coloured areas grow too rapidly to allow three successive accurate readings to be taken but during the later measurements the area does not alter appreciably in the time required.

Influence of the concentration of diffusing factor on the rate of spread of haemoglobin through the dermal tissues. From an aqueous solution (10 mg. per ml.) of a dry preparation of diffusing factor a series of tenfold dilutions was prepared, the diluent being in each case an isotonic solution of haemoglobin. Dilutions ranging from 10^{-1} to 10^{-6} were injected into the flanks of three rabbits. Two controls of haemoglobin alone were also injected. The average rate of spread of the different coloured areas is shown in Fig. 1. The area of the lesions is consistently proportional to the concentration of diffusing factor.

Reproducibility of the values. Into one flank of each of three rabbits a series of fivefold dilutions (in isotonic haemoglobin) of a solution containing the active material was injected. The same dilutions were injected into the opposite flank of the same rabbits. The rate of spread of the pigmented areas is shown in Fig. 2. The lines representing consecutive fivefold dilutions are not constantly arranged according to their concentration. When three rabbits are used the experimental error does not allow one to distinguish between fivefold dilutions. The limit of accuracy of this method appears to be tenfold dilutions.

The haemoglobin disappears from the skin in about 3 days. Identical injections were repeated after an interval of 4 days in the same areas of the animals which had been used in the previous experiment. The area and rate of spread of the coloured areas were much smaller and less regular than after the first injections. It is therefore inadvisable to use the same rabbits twice.

Activity of the standard preparation. A dry preparation obtained by a method similar to that described by Morgan & McClean [1932] is used as a standard. Serial tenfold dilutions of a solution (1 mg./ml.) of this preparation were made. Nine consecutive dilutions and a control of haemoglobin solution alone were injected into the flanks of a group of five rabbits. The lowest dilution contained 10^{-4} g./ml. and the highest 10^{-12} g./ml.

An average of the results obtained in the five animals was plotted graphically (Fig. 3). It will be seen that no definite diffusion was shown by solutions containing less than 10^{-8} g./ml. 0·01 μg. is the minimal diffusing dose of the standard preparation. 1 μg. of this powder is considered as a unit, and the activity of any preparation expressed in units per mg. or ml. Thus the standard preparation has one thousand units per mg.

Titration of a solution of unknown activity. Injections of a series of five successive tenfold dilutions of a solution of the standard preparation were made in one flank of each of a group of three rabbits. The strongest solution contained one thousand units per ml. (1 mg./ml.). Tenfold dilutions of the solution of unknown activity were injected into the other flank of the rabbits. The results are shown in Fig. 4. Dilutions of 10^{-1}, and 10^{-2} and 10^{-3} of the unknown solution produced coloured areas of similar size and rate of spread to those produced by the standard containing 10, 1 and 0·1 units per ml. Thus the diffusing activity of the solution under test was equivalent to 100 units per ml.

Concentration of the diffusing factor. The starting material used was either the testicles of recently killed bulls or a desiccated bull's testicle powder supplied by the Instituto Biologico Argentino, Buenos Aires.

Twenty-four fresh bulls' testicles (10 kg.), freed from the adjacent membranes, were minced in a meat mincer and the resulting pulp extracted with water (10 l.). Alternatively the dry testicle powder (1·5 kg.) was directly extracted with water (10 l.). After standing in the cold room (+5°) for 24 hr. the water was decanted off and the residue re-extracted three times more in a similar way.

A saturated solution of neutral lead acetate (1 l.) was added to the combined crude extract (40 l.) and the inactive precipitate removed. To the clear filtrate more neutral lead acetate (500 ml.) was added and then the solution made alkaline (*p*H 8·9) with ammonia. The active precipitate was collected. It was suspended in water (1 l.) and acidified (*p*H 4) with acetic acid. After removing the insoluble residue the Pb was precipitated by H_2S. When precipitation was complete the mixture was filtered and the filtrate concentrated *in vacuo* (bath temperature 37–40°) to half its original volume (500 ml.). Addition of acetone (4 l.) precipitates the active material quantitatively. The precipitate was extracted with water (50 ml.) and reprecipitated with acetone (500 ml.), the active

constituents now separating as a thick oil. This was almost completely soluble in water (50 ml.) and after removing a small amount of insoluble material the active constituents were precipitated as a white amorphous powder (4 g.) by addition of alcohol (250 ml.).

On account of the inaccuracy of the biological assay, even with the improvements described in this paper, it is difficult to obtain a precise idea of the concentration reached. The starting material (1500 g. of dry testicle powder) had some 10 units per mg. and the purified powder had 1000.

Purified preparations of diffusing factor are very readily soluble in water and slightly hygroscopic. They give a positive biuret reaction and a precipitate with trichloroacetic acid. The average percentage composition of the concentrate is C, 37·5%; H, 6·5%; N, 6·6%; O (by difference) 58·4%; P, 2% and 9% ash. They are completely soluble in 50% alcohol and partially in 70%, in the latter case only traces of diffusing activity going into solution. Soluble in glacial acetic acid, they are insoluble in all other non-aqueous solvents tried.

By addition of alcohol to a dilute solution of purified diffusing factor and keeping the solution for 3 months at room temperature an inactive crystalline material is obtained. The same inactive substance could be isolated from active preparations by sublimation *in vacuo* (200–220°/0·5 mm.). Recrystallised by adding an excess of alcohol to a concentrated aqueous solution or from hot 50% acetic acid it had M.P. 222–225°. (Found: C, 39·7; H, 6·8; $C_6H_{12}O_6$ requires C, 40·0; H, 6·7.) The substance showed all recorded properties of *meso*inositol and a mixed M.P. with an authentic specimen of this substance gave no depression.

Effect of the p*H on the activity of solutions of purified preparations of diffusing factor.* A solution (2 mg./ml.) of a purified preparation of diffusing factor (1000 units per mg.) was distributed in a series of tubes and each sample diluted with an equal volume of a 0·2 *M* buffer solution of the required *p*H. After 16 hr. at room temperature a small amount of each sample (2 ml.) was buffered with a 0·15 *M* phosphate buffer (*p*H 7·2, 8 ml.) and the resulting solution diluted ten times with isotonic solution of haemoglobin.

Into one flank of each of three rabbits the solutions which had been in acid medium were injected. A further three rabbits were used for the solutions which had been in an alkaline medium. The other flanks of the rabbits were injected with dilutions of the standard preparation having 100, 10, 1 and 0·1 units per ml. Control injections of haemoglobin alone were also made.

The rates of spread of haemoglobin injected with the solutions of diffusing factor which had been kept at *p*H 2, 3 and 14 were almost identical with that of the control injection of haemoglobin alone. The rate of spread of the one kept at *p*H 11·5 was similar to the one of the standard having 1 unit per ml. At *p*H 4, 5, 6, 8, 9 and 10 no destruction of activity was noticed, the haemoglobin spreading at the same rate as the solution of the standard preparation having 10 units per ml.

Effect of heat on the activity of solutions of purified preparations of diffusing factor. Two samples of a solution (1 mg./ml.) of a purified preparation (1000 units per mg.) of diffusing factor were heated at 60° and 80° respectively. Samples were taken out at 5, 15, 30 and 100 min. After cooling them as quickly as possible they were kept about 20 hr. in the cold room (+5°). Each sample was diluted ten times with an isotonic solution of haemoglobin and injected into one flank of two groups of three rabbits, each group corresponding to one temperature. The other flank of each rabbit was injected with dilutions of the standard preparation.

The rate of spread produced by the sample heated 5 min. at 80° indicated an activity corresponding to 0·1 unit per ml., whereas the unheated injected solution corresponded to 100 units per ml. Although 99·9 % of the activity was lost on heating the increase in the rate of spread of the area coloured by haemoglobin produced by the heated solution was nearly half of the increase produced by the unheated one which was a thousand times more active. 99 % of the activity of the sample heated at 60° was lost in 5 min. and about 99·9 % in 15 min.

Another sample of a similar solution (1 mg./ml.) of the same preparation (1000 units per mg.) of diffusing factor was kept in an incubator (37°) for one day. The activity of this heated solution was found to be nearly the same as that of the standard preparation (1000 units per mg.).

Summary

1. A new procedure is described for the biological assay of preparations of diffusing factor from mammalian testicle. This method, in which haemoglobin is used as an indicator, enables successive tenfold dilutions of diffusing factor to be differentiated with certainty.

2. An improved method for concentrating the diffusing factor is described, and preparations have been obtained, 0·01 μg. of which caused marked increase in tissue permeability.

3. From highly active preparations *meso*inositol has been isolated; it has no diffusing activity when tested biologically.

4. The activity of concentrates of diffusing factor is rapidly destroyed at temperatures above 37° and at *p*H below 4 or above 10.

The author's thanks are due to Dr A. R. Todd and Dr D. McClean for their helpful suggestions and to Prof. R. Robison for his valued criticisms of testing methods.

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CCXXXVI. ELECTROPHORESIS OF PEPSIN

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The first investigation of the behaviour of pepsin in an electric field was published by Michaelis & Davidsohn [1910]. They attempted to determine the sign of the charge on commercial pepsin ("purissimum Grübler") in the *p*H range 1·0–4·3, using the well-known Michaelis transference apparatus. After passing current through the apparatus, qualitative tests were made for pepsin in the compartments above the stopcocks. They found that pepsin is negatively charged at $p\mathrm{H} > 3{\cdot}7$, positively charged at $p\mathrm{H} < 2{\cdot}0$, and within this interval shows migration towards both sides. This latter phenomenon was also observed at very acid reaction (0·1 *N* HCl). The isoelectric point, obtained by taking the average of the hydrogen ion concentrations corresponding to the *p*H limits mentioned, is given as $c\mathrm{H}^+ 5{\cdot}5 \times 10^{-3}$, or $p\mathrm{H} = 2{\cdot}25$.

Since, according to later investigations of the electrophoresis of proteins, mobility is a linear function of *p*H and not of $c\mathrm{H}^+$ in the neighbourhood of the isoelectric point, it would seem preferable to take the average of the *p*H values, which would give *p*H 2·85 for the isoelectric point.

Ringer [1915], using a similar apparatus but a purified pepsin preparation, obtained somewhat different results: the enzyme showed anodic migration even in the most acid solutions and consequently had no isoelectric point. Addition of protein decomposition products (pepton Grübler), however, caused a partial cathodic migration in acid solution, which was interpreted as due to partial combination with the enzyme. On the other hand the electrochemical properties of crystalline pepsin, first prepared by Northrop, again indicate an isoelectric point of 2·8 (solubility minimum, titration curve, electrophoresis of particles of finely ground crystals) according to observations by Northrop himself [1930]. Ågren & Hammarsten [1937] in a recent paper made some interesting measurements on the electrophoresis of solutions of a crystalline pepsin preparation in the apparatus of H. Theorell. At *p*H 2·27 and 2·30 they found positive migration, at 3·34 negative, which would also indicate an isoelectric point around 2·8. A certain separation of peptic activity and total nitrogen was observed at *p*H 3·34, but at more acid reaction both migrated together. However, in recent measurements with a different material Ågren found only negative migration for pepsin (private communication).

The question of the electrophoretic homogeneity of pepsin was dealt with in considerable detail already by Ringer in his work referred to above, which is interesting because it also seems to be the first attempt to use electrophoretic methods for the study of enzymic homogeneity and for purification of enzymes (ptyalin and pepsin). Ringer worked with pepsin prepared, according to Pekelharing, from dog's gastric juice, by dialysis and by precipitation with ammonium sulphate. To explain why the pepsin showed no isoelectric point, but yet had a marked *p*H-solubility minimum, Ringer assumed that the enzyme

was partially combined with protein, which would show positive charge at acid reactions. Electrophoresis in 0·025 *N* HCl gave, in fact, an anodic portion of high activity, whereas the cathodic portion was inactive but nevertheless contained much protein. At *p*H 4·1 no such separation was observed. As regards the dependence of the separation on *p*H Ringer's results are therefore contrary to Hammarsten's and Ågren's.

The following is an account of some experiments on the electrophoretic behaviour of solutions of crystalline pepsin. The apparatus and procedure earlier described by one of us [Tiselius, 1937, 1] were used. This method has several advantages, e.g. diminished risk of heat convection, possibility of simultaneous analytical and optical observation of the migration, and of more effective separation by applying higher voltage and the "compensation" device for balancing the migration.

A number of different crystalline pepsin preparations were investigated, which will be designated below by I, II, III*a* and III*b*. Pepsin I had been prepared according to Philpot's modification of Northrop's procedure [Philpot, 1935; Philpot & Small, 1938] and formed beautiful crystals about 0·5 mm. in length. Pepsin II was a glycerol solution of crystals (twice crystallized Cudahy Pepsin 12-7-3) prepared in Dr Northrop's laboratory, according to the procedure developed there. Philpot & Small (unpublished) have found that pepsin can be fractionated by salt precipitation to give an easily soluble crystalline fraction, and a less soluble non-crystalline fraction with a solubility minimum at *p*H 3·8, the latter having a somewhat lower specific activity (private communication of the authors). Samples of these two preparations dissolved in acetate buffer were kindly provided by Dr Philpot, and will be designated below as III*a* and III*b* respectively. The authors wish to express their thanks to Drs Northrop, Philpot and Small for generously placing material and information at their disposal.

The crystals were dissolved in the buffer solution to be used; the other preparations were first dialysed to remove glycerol. Before filling the apparatus all samples were dialysed overnight against the buffer to be used as a supernatant. Nitrogen was determined by the micro-Kjeldahl method, peptic activity by the haemoglobin method [Anson & Mirsky, 1932]. Like Philpot & Small [1938] we have found that the absolute values of specific activities by this method are somewhat dependent upon the preparation of haemoglobin used. Throughout this work, therefore, a single haemoglobin preparation was used.

Preliminary electrophoresis measurements showed that the various preparations contained one definite protein component of fairly homogeneous migration, with only small differences in migration velocity. Besides the sharp boundary given by this component, a very diffuse boundary could be observed, at least in concentrated solutions (more than 0·5%), indicating the presence of inhomogeneous material.

The final experiments involved optical determinations of the mobility of the main component at different *p*H (Table I, Fig. 1); in a number of experiments the optical observation was supplemented by analysis of the different compartments for nitrogen and peptic activity (Table II, Fig. 1), in order not only to correlate the activity migration with the migration of the main component but also to see how far the specific activity could be raised by electrophoretic separation. As the "analytical" experiments for this latter purpose were usually run for a much longer time, a higher buffer concentration (ionic strength 0·1) was used than in the "optical" series (ionic strength 0·02). In prolonged electrophoresis experiments disturbances due to electrolyte transport occur much more easily in dilute salt media than in more concentrated solutions.

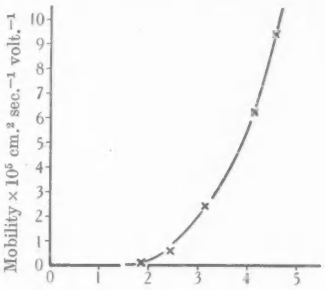

Fig. 1 *a*.

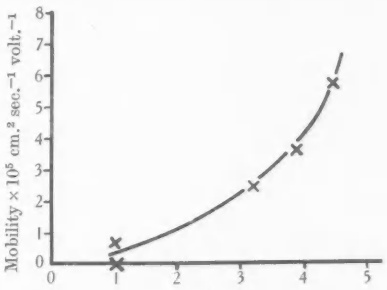

Fig. 1 *b*.

Fig. 1. Electrophoretic mobilities of the active component in pepsin at 0° and ionic strengths (*a*) 0·02 and (*b*) 0·1.

Table I. *Electrophoretic migration velocity of main component in solutions of crystalline pepsin*

Optical observation. Buffer ionic strength 0·02. Pepsin conc. 0·2–0·3 %. Temp. 0°.

Preparation of pepsin	Buffer	*p*H	Mobility × 10^5 cm.² volt^{-1} sec.$^{-1}$
I	Acetate	4·57	−9·4
III*a*, II	Acetate	4·15	−6·2
I	Citrate, HCl	3·16	−2·4
II	Citrate, HCl	2·47	−0·6
II	Citrate, HCl	1·87	−0·1

Determinations in 0·1 *N* HCl, *p*H 1·08 gave values for the mobility varying between 0·0 and $-0{\cdot}4 \times 10^{-5}$. The variation is probably due to the fact that the experimental error in this medium is much greater (too low buffering action). No positive migration could be observed in any of the preparations.

Table II. *Electrophoretic migration and fractionation of solutions of crystalline pepsin*

Optical and analytical methods used simultaneously. Ionic strength of buffer = 0·1. Temp. 0°.

					Mobility × 10^5 cm.² volt^{-1} sec.$^{-1}$			Specific activity $[P.U.]^{Hb}_{mg.\ total\ N}$	
Preparation of pepsin	Buffer	*p*H	Potential gradient volt/cm.	Time min.	Peptic activity	Total nitrogen	Main compartment (optical observation)	Original solution	Top + compartment at end of exp.
I	Citrate HCl	3·19	7·64	195	−2·68	−1·38	−2·42	0·162	0·273
I	HCl	1·08	3·06	315	±0·0	+0·6	±0·0	0·177	0·246
II	Acetate	4·41	7·65	595	—	—	−5·65	0·214	0·344
II	Citrate HCl	3·19	9·30	515	—	—	−2·40	0·213	0·324
III*a*	Citrate HCl	3·19	9·04	597	—	—	−2·50	0·240	0·314
III*b*	Citrate HCl	3·19	9·39	493	—	—	−2·32	0·175	0·250

The first two experiments in Table II were made with the purpose of measuring migration velocities by the three different methods simultaneously, whereas in the others we tried to obtain an appreciable separation by prolonged migration (the main protein component migrated from 64 to 154 mm.) in combination with compensation [Tiselius, 1937, 2]. Under these circumstances a change in the

composition of the bottom compartment was unavoidable and calculations of mobilities from analytical measurements in the usual way became impossible.

The results obtained in the experiments described in Tables I–II and Fig. 1*a*, *b* may be summarized as follows. Solutions of crystalline pepsin, prepared by various methods, were in no case found to be quite homogeneous in electrophoresis, but contained some inactive nitrogen which could be removed by electrophoretic separation. In this way the specific activities of the various samples could be raised by 31–69 %. From the agreement between the mobilities (Table II, 6th and 8th columns) it is concluded that the peptic activity is associated with a protein component, which shows fairly homogeneous migration (does not give rise to more than one "schlieren" band in the optical observation arrangement). This protein in the *p*H range investigated (*p*H 1·08–4·57) carries a negative charge and shows no isoelectric point and thus behaves electrophoretically like an acid. Even a pepsin preparation which showed a solubility minimum at *p*H 3·8 (last experiment in Table II) behaved in principle in the same way.

Our experiments thus to a certain extent give further support to the view expressed by Northrop that the enzyme pepsin is a protein, but it is quite clear that pepsin crystals contain considerable amounts of inactive material, which may be removed by electrophoresis, and it seems likely that this material influences some of the properties of pepsin, such as solubility and acid-base combining capacity, in a marked way. The specific activity of crystalline pepsin according to Anson & Mirsky is 0·184 $[\text{P.U.}]^{\text{Hb}}_{\text{mg. protein N}}$, but in a recent publication by Herriot [1938] a higher value is given (0·26). We have preferred to refer our values to total nitrogen instead of protein nitrogen and (as seen from Table II) we obtained activities ranging from 0·162 to 0·240 for our starting material, but as high a value as 0·344 $[\text{P.U.}]^{\text{Hb}}_{\text{mg. total N}}$ for our most active fraction. In comparing these values with those obtained by other authors one must make the reservation that the haemoglobin preparations were not quite identical [see also Philpot & Small, 1938].

As regards the question of the homogeneity of pepsin it should be observed that the electrophoretic procedure is a very gentle separation method and could not break down a loose association complex, unless this were appreciably dissociated into its components. The homogeneity of the migration of the active component as found in the experiments described above is not therefore sufficient evidence *per se* for the chemical individuality of this enzyme, especially as the most active fractions obtained from different materials did not show the same specific activity (Table II). More experiments over a wider *p*H range and with systematically varied preparation methods are needed to clarify this question.

The somewhat surprising result that pepsin is not positively charged even in the most acid solutions investigated (*p*H 1·08) may possibly be due to an unusual tendency of the pepsin to combine with anions of the electrolyte medium. Such an effect has been previously found [Tiselius, 1930] for egg albumin, although not to as marked an extent as one would have to assume for pepsin to account for the above results. This would explain why, in the most acid solutions, the negative charge is larger when the ionic strength increases from 0·02 to 0·1 (see Fig. 1), contrary to the usually observed decrease in electrophoretic mobility with increasing salt concentration.

SUMMARY

Electrophoretic measurements show that the enzymic activity of solutions of crystalline pepsin, prepared by different methods, migrates as a homogeneous protein component with a negative charge and with no isoelectric point. Some inactive, inhomogeneous protein or protein breakdown material is left behind, and the specific activity may thus be raised by 31–69 %.

The authors are indebted to Prof. Svedberg for his kind interest in this investigation, and to the Nobel, Rockefeller, and Wallenberg Foundations for financial support.

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CCXXXVII. THE FERMENTATION PROCESS IN TEA MANUFACTURE

I. THE ROLE OF PEROXIDASE

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The fermentation of the tea leaf is a stage in the manufacturing process of tea which has received but little attention in the past. Various qualitative studies have been made at the several Tea Research Institutes but no thorough investigation has been made apart from that of Oparin *et al.* [1935].

Results in this field have a considerable commercial use and they are also of interest to the biochemist, not only as an illustration of the growing importance of enzyme chemistry in industry but also as a detailed study of the chemical changes taking place in damaged plant tissue.

Before dealing with the fermentation process itself it may be of advantage to outline the various stages in tea manufacture.

After plucking, the leaf is spread out in thin layers to undergo withering. During this process which usually takes about 18 hr. the leaf loses water and becomes flaccid. Unless this condition of flaccidity is produced the leaf is broken into flakes, instead of being twisted in the subsequent rolling process, and the resultant dry tea loses market value on account of its appearance. Not only is the withering process a physical preparation of the leaf for the further stages of manufacture but also some chemical changes may take place which partly determine the final quality of the manufactured tea. The nature of these changes however is still obscure.

The next stage in manufacture is rolling. The primary object of this process is to rupture the leaf-cells and to expose the expressed juices to the air. At the same time the tea receives its characteristic twist. As soon as the leaf tissue is in any way damaged fermentation starts, so that it is impossible to separate the rolling process from the next stage which is that of fermentation. After a period of about $1\frac{1}{2}$ hr. rolling the leaf is spread thinly on a fermenting floor, usually of polished concrete, where the oxidation process which commenced in the rollers is continued for perhaps another 2 hr., after which the tea is fired to stop fermentation and to dry the leaf.

During fermentation oxidative changes take place which largely determine the flavour and colour of the infused liquor. The amount of tannin extractable by hot water is considerably reduced, and some of the colourless tannin is transformed into red and brown products which arise from the original tannin, partly by oxidation and possibly partly by condensation. The precise mechanism of these changes is not yet properly understood.

Theories of the fermentation reaction

Although the fermentation process has been ascribed to simple oxidation, and, at a later date, to the activity of enzymes of micro-organisms on the leaf, there has been very little doubt since the work of Mann [1901; 1903; 1904] that

the oxidation is a process catalysed by the peroxidases of the leaf itself. The fermentation process is but another example of the general post-mortem changes that set in after damage to plant-tissues.

Fermentation will not take place *in vacuo* but starts as soon as oxygen is admitted into the system. The oxidative process takes place only within certain limits of acidity, the *p*H optimum being about 5–6. Acidification stops fermentation, while addition of alkali accelerates a non-enzymic autoxidation of the tannin bodies. Small amounts of KCN completely inhibit fermentation, as does a preliminary steaming of the leaf. All these observations are characteristic of an enzymic oxidation, and proof of this is afforded by the isolation of a peroxidase from the leaf which rapidly browns a green leaf infusion in the presence of H_2O_2 as is described later in this communication.

While there can be very little doubt that the oxidative process is catalysed by enzymes of the leaf, there is no doubt that micro-organisms do exert a very considerable influence on the quality of the final product. As yet only a lowering of quality is recognized as a result of the uncontrolled development of micro-organisms during fermentation, but this does not exclude the possibility that there may be some beneficial results.

Nature of the enzyme and substrate in the tea leaf

Having established with reasonable certainty that the fermentation of tea is an oxidative process catalysed by enzymes, the first thing to establish is the nature of the enzyme involved.

Using a press juice from fresh green leaf, artificial substrates such as the "Nadi" reagent do give a positive reaction for oxidase, but the development of colour as seen in a toluene extract is very much intensified by the addition of H_2O_2. If the leaf is ground up with sand under alcohol the oxidase reaction becomes negligible while peroxidase activity is intensified. This intensification of peroxidase activity which is due to the removal of tannin will be discussed later. The disappearance of a weak oxidase reaction on washing with alcohol is probably due to removal of H_2O_2 formed in the leaf, small amounts of which are to be expected. Its presence would be revealed by a slight peroxidase reaction without the addition of further H_2O_2. As a result, therefore, of qualitative tests oxidase cannot be detected in the tea leaf, while peroxidase activity is very marked. This confirms the observations of Manskaya [1935] who ascribed the oxidative processes in the tea leaf to peroxidase action; oxidase could only be found in negligible quantities. Any mechanism for the fermentation process must take account of this quantity of peroxidase and also a reaction involving oxygen uptake not catalysed by polyphenol oxidase must take place, as fermenting leaf takes up a considerable amount of oxygen.

The work of Szent-Györgyi [1937] and Huszák [1937] makes it very probable that in peroxidase plants oxygen is transferred to metabolites by the following reactions.

$$\text{Ascorbic acid} + O_2 \xrightarrow{\text{ascorbic acid oxidase}} \text{dehydroascorbic acid} + H_2O_2$$

$$H_2O_2 + \text{catechol derivative} \xrightarrow{\text{peroxidase}} o\text{-quinone}$$

$$o\text{-Quinone} + \text{ascorbic acid} \longrightarrow \text{dehydroascorbic acid.}$$

In normally respiring plant tissue the oxidation products are again reduced by activated H (dehydrogenase activity). It seems quite probable that the irreversible oxidations of damaged plant tissues are due to a dispersal of reducing

substances and coenzymes. Under these conditions the oxidation products formed in the above reactions find no activated H available and consequently undergo further transformations to form deeply coloured oxidation products of catechols.

It seems therefore very reasonable to suppose that in the fermentation of tea oxygen is absorbed, one of the products of oxidation being H_2O_2, and that the tea tannin is then oxidized by the H_2O_2 with the catalytic assistance of the peroxidase. This theory has been tested experimentally and found to be absolutely correct.

Experimental

If tea leaf is finely minced it undergoes the fermentation reaction very readily even when suspended in water. If such a suspension of minced tea leaf is kept *in vacuo* no browning of the tissues is apparent for several days, whereas on exposure to oxygen the leaf assumes a brownish tinge throughout in 2 to 3 hr. If dilute H_2O_2 (such that the final concentration of H_2O_2 does not exceed $N/50$) is added slowly to the minced leaf suspension, stored *in vacuo*, fermentation starts at once, and the suspension soon becomes uniformly brown. Anaerobic fermentation is therefore possible in the presence of H_2O_2. The same experiment shows that fermentation is probably catalysed by peroxidase.

It should be possible by mixing tea tannin with peroxidase and H_2O_2 to obtain the same chemical changes that occur in the actual fermentation process. Using a specimen of tea tannin kindly provided by Mr C. J. Harrison, and a tannin-free peroxidase extract from green tea leaf, the fermentation changes could not be simulated. Tannins, although tea tannin is not a true tannin, are known to have an inhibiting effect on peroxidase so that it was thought that the reason for this failure might be due to inactivation of the enzyme. Using a green leaf infusion instead of tea tannin, results indicating a satisfactory degree of oxidation were obtained. The infusion obviously contains substances which protect the enzyme from destruction by tea tannin.

On mixing the infusion, which, having been boiled, is free from enzymes, with a peroxidase solution, fermentation proceeds very slowly indeed, but on the addition of H_2O_2 the development of the brown fermentation colour is almost immediate. The optimum concentration of the H_2O_2 is about $N/200$. At higher concentrations the rate of fermentation is slowed down considerably and may even cease. To obtain the deepest brown colours it is best to add the H_2O_2 in small quantities at a time, never allowing it to accumulate in the system. This is in accordance with the well known susceptibility of peroxidase to H_2O_2 established by Willstätter & Weber [1926], Guthrie [1931], Mann [1931], and many others. H_2O_2 alone fails to oxidize tea tannin.

Tea tannin however only accounts for about 20% of the solid matter in a green leaf infusion, and the proper development of the fermentation colour in the experiment just described, while establishing the participation of peroxidase and H_2O_2 in the fermentation process, does not prove that it is the oxidation of the tannin to which this brown colour is due. Zender [1925] instances the oxidation of true tannins by oxidases so tea tannin is a likely substrate for peroxidase.

The same experiments were therefore carried out quantitatively, the tannin being titrated by $KMnO_4$ according to Löwenthal's method as modified by Carpenter & Harler [1922]. Incubation of a tea leaf infusion with peroxidase and H_2O_2, added in successive small quantities, materially decreased the tannin titre, any excess H_2O_2 remaining being decomposed by the catalase also present in the peroxidase solution. Oxidation appeared to come to a standstill after the

addition of a certain amount of H_2O_2 (Table I), but the amount oxidized was increased either by doubling the amount of enzyme or by adding further enzyme and H_2O_2 when the oxidation was apparently complete (Table II). The apparent completion of oxidation was therefore due to destruction of the enzyme in the course of the fermentation, a point of practical importance when considering the optimal time of fermentation in the manufacturing process.

It will be noted, from the results of Table II, that when peroxidase is in excess, the decrease in the tannin titre is greater than that which corresponds to the amount of H_2O_2 added. This has been observed on each occasion that excess peroxidase was present, and it would seem to indicate that the enzymic oxidation product of tannin can undergo further oxidation without the assistance of H_2O_2. It is well known that the oxidation of catechol derivatives rarely stops short at the first stage of oxidation, and subsequent stages of oxidation may take place readily without the aid of either enzyme or H_2O_2.

$KMnO_4$ titration is not specific for tea tannin in an infusion and it is usual to subtract from these figures the results obtained for non-tans. The tannin, in a larger aliquot portion than that used for crude tannin estimation, is precipitated with gelatin, precipitation being aided by salting out with acid and NaCl. The $KMnO_4$ titre of an aliquot part of the filtrate gives the non-tan value. Determinations of crude tannin and non-tan after incubation with peroxidase and H_2O_2 show that non-tans are also oxidized to much the same extent as tea tannin itself (Table III). These non-tans may of course be catechol bodies. In these experiments a filtered peroxidase solution was used, as it was found that the end point with indigocarmine was much sharper than with the unfiltered solution.

Table I

ml. $N/20$ H_2O_2 added	Crude tannin titration ml. $N/20$ $KMnO_4$	Difference
—	9·45	—
2·0	7·65	1·80
4·0	6·20	3·25
6·0	6·05	3·40
8·0	5·85	3·60

An infusion of 10 g. green leaf in 100 ml. of water and a peroxidase extract of 100 ml. from a further 10 g. of leaf were prepared according to the directions given later in this paper. 10 ml. portions of these solutions were mixed and 0·5 ml. portions of $N/20$ H_2O_2 added at intervals of 10 min. After addition of the H_2O_2 the solution was titrated with $N/20$ $KMnO_4$ in the presence of indigocarmine.

Table II

		Fall in tannin ml. $KMnO_4$
A.	5 ml. infusion + 5 ml. peroxidase + 2 ml. $N/20$ H_2O_2	2·0 ml. $N/20$ $KMnO_4$
B.	5 ml. ,, + 10 ml. ,, + 2 ml. ,,	4·2 ,,
C.	5 ml. ,, + two successive 5 ml. portions peroxidase and 2 ml. portions H_2O_2	5·0 ,,

Table III

	Tannin ml. $N/20$ $KMnO_4$	Non-tan ml. $N/20$ $KMnO_4$
Initially	7·5	4·55
At end of fermentation	4·8	2·1

5 ml. each of infusion and enzyme are mixed and four portions of 0·5 ml. $N/20$ H_2O_2 are added at 10 min. intervals. After the last addition another 5 ml. of enzyme and four more portions of H_2O_2 are added. For non-tan titrations five times these quantities are used.

In the actual fermentation process in manufacture both tannins and non-tans undergo oxidation but the oxidation of non-tan is relatively much less.

Table IV

	% Tannin	Non-tan*
Withered leaf	21·45	6·5 ml. $N/20$ $KMnO_4$
Leaf after 5 hr. rolling and fermentation	13·20	5·1 ,,

* As non-tans are ill-defined substances which have not as yet been separated they cannot be expressed on a percentage basis. They account for as much as 40% of the total permanganate titre of a tea extract. Shaw [1930] however is of the opinion that, in the gelatin precipitation, part of the tea-tannin molecule is split off and remains in the filtrate to be estimated as a non-tan.

The role of hydrogen peroxide

Reactions exactly analogous to those occurring in the fermentation process have been carried out *in vitro*, as just described, and the conclusion may be drawn that the browning of the tissue is due to peroxidase action on the tea tannin. A point unsettled in these experiments however is the participation of H_2O_2. This substance can be found only in very small quantities indeed in the fermenting leaf, but as it would be continually used up by peroxidase as soon as it was produced, and as the catalase present effectively checks its accumulation, direct methods of estimation would not be expected to reveal its presence.

In the past in discussing the role of peroxidase in plant respiration there has been considerable talk of the possibility of organic peroxides taking the place of H_2O_2 in peroxidase catalysed reactions. This is the theory adopted by the Russian workers in this field throughout the whole of their work.

There is no experimental evidence in favour of this view, in fact all the available evidence contradicts it. Wieland & Sutter [1930] found that mono-substitution of the H atoms in H_2O_2 by organic radicals materially reduced its ability to function as an oxidizing agent in the presence of peroxidase. Disubstituted products were completely without effect. Böeseken [1930] confirmed these results and added organic per-acids to the list of substances incapable of replacing H_2O_2. Szent-Györgyi [1925] has shown that *o*-quinones oxidize guaiacum without enzymic aid and we have shown that not only do *p*-quinones oxidize the Nadi reagent much more rapidly than the equivalent concentration of H_2O_2 in the presence of peroxidase, but that this oxidation by quinone is not accelerated by peroxidase. As there are several processes that can result in the formation of H_2O_2 in the leaf, and as the presence of catalase is a further indication of its presence, it will therefore be assumed that peroxidase oxidations in fermentation do actually utilize H_2O_2 and not some unspecified peroxide. The participation of H_2O_2 in the fermentation process is however capable of experimental verification. Catalase is a completely specific enzyme and if it could be shown that tannin oxidation is inhibited by catalase, it would prove that H_2O_2 is concerned in the fermentation process.

As shown before suspensions of minced leaf in water undergo fermentation. If excess of a liver catalase preparation is added to this suspension, fermentation is almost completely inhibited. If the catalase preparation is first inactivated by heat, fermentation proceeds unaffected.

It may therefore be concluded that H_2O_2 is concerned in the fermentation of tea, and as this fermentation is accompanied by a considerable uptake of O_2 it seems reasonable to ascribe the formation of H_2O_2 to this process. The nature of the O_2 uptake and the origin of the H_2O_2 will be investigated at a later date.

The oxidation of tannin is therefore a coupled oxidation such as that first established by Thurlow [1925]. Here secondary oxidation of guaiacum tincture by peroxidase was brought about in the presence of the H_2O_2 developed during the aerobic oxidation of hypoxanthine by xanthine oxidase. Kursanov's views [1935, 2], with the exception that he considers organic peroxides to be produced and not H_2O_2, are identical with ours. The absorption of oxygen and the production of H_2O_2 whether it is enzymic or not is preliminary to fermentation and does not lead to any development of colour.

Estimation of peroxidase activity in tea leaf

Having proved the importance of peroxidase in fermentation it becomes necessary to have some method of determining its activity in the tea leaf. The purpurogallin method, although the simplest, cannot be used in Assam throughout the whole year owing to the volatility of ether in the hot weather. The method of Guthrie [1931], with suitable modifications, proved to be the best available.

Technique and preparation of substrate and enzyme

The substrate was made up exactly according to Guthrie. 2·1 g. cryst. citric acid were dissolved in 17 ml. *N* NaOH and diluted to 100 ml. To 50 ml. of this solution 50 ml. of water were added 0·25 g. of *p*-phenylenediamine hydrochloride and 5 ml. of a 4 % solution of α-naphthol in 50 % alcohol. The solution was then filtered. 10 ml. of this reagent were then mixed with the enzyme solution (usually 2 ml.) and 2 ml. of *N*/20 H_2O_2 were then added. After 10 min. incubation the reaction was stopped by the addition of 2 ml. 1 % KCN. An aliquot portion, usually 3 ml., was taken and extracted with 25 ml. toluene and the depth of colour in the toluene layer estimated in a Lovibond tintometer. By a suitable mixture of reds and blues the colour could be matched exactly. A solution is said to have 6 Indophenol Units (I.U.) when the total reading of the red and blue slides is 6·0. The readings of the tintometer are not directly proportional to the concentration of indophenol blue, but a smooth calibration curve can easily be constructed. Determinations are accurate and reproducible to 0·1 I.U.

Tea juice or a suspension of the finely ground leaf in water cannot be used as the source of the enzyme as peroxidase activity is inhibited by tannin. Pretreatment of the leaf with alcohol seems to have no destructive effect on the enzyme, so the leaf sample is first ground up finely with sand under alcohol. The ground up tissue is filtered at the pump and the residue washed with alcohol until the washings are colourless. When all the chlorophyll has been removed the washings no longer show a positive tannin reaction.

After rapid drying *in vacuo*, to remove alcohol, the powdered residue is extracted with *M*/15 phosphate buffer (*p*H 7·0). A convenient enzyme solution is obtained by extracting the ground up residue from 10 g. leaf with 100 ml. phosphate. 1 ml. of the extract is then proportional to 0·1 g. of the original leaf. For determinations on single shoots (two leaves and a bud) the residue was extracted with 10 ml. phosphate, and an aliquot portion of this, 3–5 ml., was used in the estimation.

Testing of the method

The method described above must satisfy two conditions, the peroxidase must be extracted quantitatively from the leaf, and the amount of indophenol blue formed must be proportional to the enzyme concentration. That these conditions are fulfilled is shown in the following experiments.

Alcohol has very little effect on the activity of a peroxidase preparation even after 3 days' contact. Extraction of the peroxidase with the phosphate is quantitative, as if the tannin-free residue from 1 g. of leaf is extracted with 10 ml. of phosphate the activities of a 5 ml. portion of this extract and of the remaining 5 ml., together with the residue, are identical. Further, extractions for 5 and 15 min. gave enzyme solutions of identical activity.

The peroxidase activity of the final extract is much higher than that of an extract of fresh leaf owing to the tannin content of the latter. If to the final extract an amount of tea tannin equal to that originally present in the fresh leaf is added, it is found that the peroxidase activity of the extract is now depressed to the value it had originally in the fresh leaf. This has been established using both the indophenol blue and purpurogallin methods.

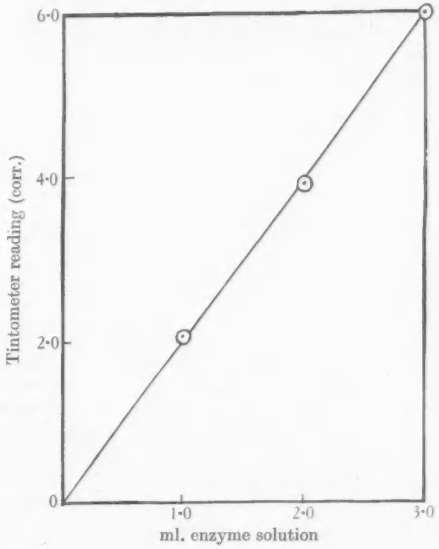

Fig. 1.

These experiments are considered to be sufficiently conclusive for it to be taken that extraction of the enzyme is quantitative.

The second requirement, that the extent of indophenol blue formation is proportional to enzyme concentration, is also fulfilled. The curve (Fig. 1) shows that the depth of colour is strictly proportional to enzyme concentration.

It is hoped to report the results obtained with this method in the study of fermentation at a later date.

Inhibition of peroxidase by tea tannin

Juice expressed from fresh tea leaf does not blue guaiacum even in the presence of H_2O_2. It was suspected that this was due to the inhibiting effects of tea tannin, and confirmation of this was found by showing that the addition of tea tannin inhibited the blueing of guaiacum by potato juice. If the tannin

is removed from the tea leaf by extraction with alcohol a positive peroxidase reaction is then given with guaiacum.

Tea tannin inhibits the formation of indophenol blue and of purpurogallin by peroxidase, but apparently to different extents. Table V gives the relative amounts of these two pigments formed in the presence of varying amounts of tea tannin.

Table V

	Indophenol blue	Purpurogallin
2 ml. enzyme	14·5	16·0
,, +2 ml. 0·05% tea tannin	7·6	14·1
,, +2 ml. 0·5% ,,	1·9	14·0
,, +2 ml. 5% ,,	0·5	9·5

The very marked inhibition of the indophenol blue reaction is partly apparent as tea tannin combines with indophenol blue and is not then extractable by toluene. If the tea tannin is added after the reaction has been stopped by KCN, most of the indophenol blue is fixed by the tannin and comparatively little is extracted by toluene. Acids have a similar effect so that the indophenol reaction must never be stopped by addition of acid as the weakly basic dyestuff is then retained in the aqueous phase on extraction with toluene. The inhibition of purpurogallin formation is a true enzymic inhibition; addition of tea tannin, after stopping the reaction, has no effect on the amount of purpurogallin formed.

In the above table, when 2 ml. 0·5% tannin are added to the enzyme extract, tea tannin and peroxidase are in approximately the same proportions as they are in the leaf. The importance of removing tea tannin before estimating the peroxidase activity of a sample of leaf is at once apparent. True inhibition by tannin is not very high at this concentration but the degree of combination with indophenol blue is sufficient to make the results highly erroneous.

The theory has been advanced by Kursanov & Oparin [1929] that the inactivation of peroxidase by tea tannin can be minimized by protein decomposition products. In the leaf these were supposed by Kursanov [1935, 1] to originate by proteolysis during the withering stage of the manufacturing process. There seems no doubt that a green leaf infusion does contain protective substances as such an infusion is oxidized by peroxidase whilst tea tannin itself is not. On the other hand the evidence that these protective substances are protein decomposition products is slight.

To investigate this possibility experiments have been made using bacteriological peptone, which gives a precipitate with tea tannin, and a mixture of amino-acids. Neither had any effect over a whole range of concentrations on the formation of indophenol blue in the presence or absence of tannin. On the other hand both substances inhibited purpurogallin formation, presumably by combining with the quinone initially formed from pyrogallol, but had no effect on tannin inhibition. The effects of peptone (or amino-acid) and tannin were additive.

The suggested role of protein breakdown products in promoting fermentation receives therefore no confirmation.

Variation of peroxidase throughout manufacture

Some preliminary results are recorded here on the variation in peroxidase content of the leaf in the various stages of manufacture from green leaf to the final manufactured tea.

In each experiment 50 lb. of green leaf were manufactured. Samples were taken in duplicate of the green and withered leaf, at two different stages of rolling and at intervals throughout fermentation. When the fermented leaf was fired, part was retained to undergo further fermentation up to a period of 25 hr. The fired tea was also estimated for peroxidase activity. In cases where peroxidase activity was low, the amount of indophenol blue formed was increased either by increasing the amount of enzyme solution or by increasing the aliquot portion of the final incubation mixture. For fired tea both enzyme and aliquot had to be increased. Enzyme determinations were performed in duplicate. The sampling error was not great, only once did duplicates differ by more than 5% and this was with leaf fermented for 25 hr. where bacterial development was very marked.

For the sake of economy in space the results obtained are not quoted in full. Enzyme activities are expressed in terms of I.U. per g. of dry tissue. The results obtained for a normal manufacture and for a protracted fermentation are tabulated in Tables VI and VII respectively.

Table VI

Green leaf	894	I.U. per g. dry wt. of tissue
Withered leaf	1293	,, ,,
40 min. roll	780	,, ,,
90 ,,	685	,, ,,
2½ hr. fermentation	647	,, ,,
4 ,,	536	,, ,,
Fired tea	7	,, ,,

Table VII

Withered leaf	1051	I.U. per g. dry wt. of tissue
90 min. roll	652	,, ,,
3¼ hr. fermentation	617	,, ,,
5 ,,	178	,, ,,
6½ ,,	115	,, ,,
25 ,,	132	,, ,,

As has been noted before by Mann [1901; 1903; 1904] and Manskaya [1935] there is a definite increase in activity during withering. We also find a definite decrease in peroxidase activity during the first roll, after which the fall, although regular, is comparatively slight up to the point where fermentation is stopped by the firing process. If fermentation is prolonged beyond 4 hr. peroxidase activity again falls rapidly.

The earlier experiments described in this paper established the toxic effects of both tannin and H_2O_2 on the enzyme causing fermentation; in fact the enzyme could be completely inactivated. These factors are not so important in actual manufacture, as not only is H_2O_2 present in very low concentration but also resistance to tannin inactivation is much greater, owing presumably to similar protective bodies to those shown likely to be present in the green leaf infusion.

It is found that the firing process does not completely inactivate the peroxidase, 0·5 to 1·0% of the original activity of the withered leaf being retained. Some of this activity is retained for at least 6 months. It is suggested that this residual activity may account in part for the post-fermentative changes which occur in the maturing of tea.

Summary

A brief outline is given of the various stages in the manufacture of tea.

Oxidase is present in negligible quantities in the tea leaf and the fermentation process is brought about by peroxidase. The first stage in fermentation is an

uptake of oxygen leading to the oxidation of some as yet unidentified substance, and the production of H_2O_2. This oxidation is not necessarily an enzymic process and may be an autoxidation. The peroxidase then catalyses the oxidation of the tea tannin by H_2O_2 leading to the production of brown pigments which impart the characteristic colour to a tea infusion. Substances, grouped as non-tans, may also undergo oxidation.

No evidence is available for the utilization of organic peroxides by peroxidase, and in the fermentation of tea, decomposition of H_2O_2 by excess catalase inhibits tannin oxidation almost completely.

A quantitative method for the estimation of peroxidase in the tea leaf is described.

Peroxidase is inhibited by tea tannin although substances present in the leaf exert some protective influence against its effect. The theory that these protective substances are protein degradation products, formed during withering, finds no support.

An outline is given of the changes in peroxidase content of the leaf throughout the various stages in manufacture.

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CCXXXVIII. THE INFLUENCE OF THIOL GROUPS IN THE ACTIVITY OF DEHYDROGENASES. II

WITH AN ADDENDUM ON THE LOCATION OF DEHYDROGENASES IN MUSCLE

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It was shown [Hopkins & Morgan, 1938] that when washed tissues are incubated anaerobically for adequate periods with solutions of glutathione (GSSG) at *p*H 7·4 the succinic dehydrogenase is completely inactivated. On the other hand the original activity of the enzyme is fully regained when the preparations so treated are subsequently re-incubated with the reduced form of glutathione (GSH). In the case of a second enzyme, the α-glycerophosphate dehydrogenase, the activity is wholly unaffected by the treatment described. Hopkins and Morgan suggested that these facts show that a thiol group in its structure is essential for the activity of the former enzyme, but not at all for that of the latter.

In experiments now to be described these observations have been confirmed and extended. A number of other dehydrogenases have been studied, but among them the succinic dehydrogenase was the only one found to respond to the influence of glutathione. The influence on dehydrogenases of other substances known or supposed to inhibit the action of hydrolytic enzymes by combining or reacting with SH groups in their structure has been investigated, and also the influence of these and other inhibitors in protecting the succinic dehydrogenase from the action of glutathione. Preliminary experiments will be described which seem to bear on the location of dehydrogenases in muscle fibres.

Experimental

Methods

In most of the experiments washed muscles from the hind legs of rabbits were employed, but in a few the enzyme preparations were made from other tissues. The muscles, immediately after their removal from the animal, were passed through a Latapie mincer and washed thrice with distilled water. Occasionally they were subsequently finely ground with sand in a mechanical mortar. The effect of various forms of treatment on the activity of the enzymes was in all cases determined by the anaerobic methylene blue technique; but in the cases of the succinic, glycerophosphate and lactic enzymes the degree of activity was also determined spectroscopically by observing the rate at which a preparation, in the presence of a substrate, reduced cytochrome *c*. In some experiments with succinic dehydrogenase the rate of O_2 uptake in the presence of cytochrome and substrate was measured.

In the methylene blue experiments the procedure was made as nearly as possible the same in all experiments, so that usually in the individual protocols only the reduction times need be reported. In general 0·5 g. washed muscle was employed at this stage, or its calculated equivalent when, as a result of treatment, the tissue had gained or lost water. Small Thunberg tubes were used, and each tube contained, in addition to the tissue, 1 ml. phosphate buffer at *p*H 7·4 and 1 ml. water. To this was added 0·5 ml. *M*/2 substrate, the final concentration of the latter being therefore *M*/12. Incubation was always carried out *in vacuo* at 37°.

In all experiments which involved incubating the tissue with glutathione or other substances, the control sample used in the comparison of reduction times was itself incubated simultaneously in buffer solution without additions. The method of incubation was that described in the previous paper. The amount of methylene blue employed was uniformly 0·25 ml. of *M*/1000 solution.

I. The succinic dehydrogenase

That this enzyme can suffer complete loss of activity by adequate treatment with GSSG, and that its activity is restored on subsequent exposure to GSH solutions, has been repeatedly confirmed during our experiments. Since, as was pointed out in the previous paper, these reactions involve equilibria the complete removal of activity calls for more than one exposure to the GSSG solutions, though its complete restoration usually requires one exposure only to solutions of GSH of sufficient concentration. In many of the following experiments complete inactivation was not found necessary; wide changes in the degree of activity gave the information sought.

The suggestion has been made to us that the effect of GSSG might not involve an oxidation of SH groups as was assumed in the previous paper to occur, but might follow on the lines of some other inhibitions of enzyme activity. The constitution of the disulphide peptide is such that it might enter into a reversible association with the enzyme of a kind which would prevent essential groups in the structure of the latter from exerting their normal influence.

Such a view, unlikely from the general evidence discussed by Hopkins & Morgan [1938], seems to be disproved by experiments of the following simple kind. The process of inactivation, it will be shown, involves a reaction which has a prominent time factor and a somewhat high temperature coefficient, while its velocity increases continuously through a wide range of increase in the concentration of GSSG. In these respects it differs entirely of course from inhibition by agents which may be assumed to act by simply blocking the enzyme surface; e.g. malonic acid (see below).

Exp. 1. Latapie-minced and well-washed muscle tissue in portions of 0·5 g. was incubated anaerobically for 2 hr. at *p*H 7·4 with varying concentrations of GSSG. The samples were filtered off on a linen filter, well washed, and their reduction times determined as described above.

Exp. 1

	Reduction times
Original tissue	10 min.
Incubated with *M*/100 GSSG	17 min.
Incubated with *M*/50 GSSG	25 min.
Incubated with *M*/10 GSSG	2 hr. 30 min.

It is seen that the reduction time (inactivation of the enzyme) increases continuously with the concentration of GSSG.

Exp. 2 demonstrates the effect of incubation time on the degree of inactivation. Equal portions of washed muscle were incubated *in vacuo* for various times with 0·8 ml. *M*/5 GSSG, then filtered off and washed. One sample after incubation for 2 hr. was incubated for a second 2 hr. with a fresh supply of GSSG.

Exp. 2

	Reduction times
Original tissue	16 min.
Incubated for 25 min.	23 min.
Incubated for 60 min.	46 min.
Incubated for 2 hr.	1 hr. 30 min.
2 hr. sample re-incubated	∞

Exp. 3 shows the effect of temperature on the process of inactivation. Samples of muscle (0·5 g.) treated as before were incubated, with and without GSSG (*M*/10), for 4 hr. at different temperatures. Washed as usual.

Exp. 3

Temperature	Reduction times
20° control (no GSSG)	12 min.
with GSSG	15 min.
30° control	12 min.
GSSG	56 min.
40° control	12 min.
GSSG	2 hr. 40 min.

These three experiments show that inactivation by GSSG involves a progressive chemical reaction with a somewhat high temperature coefficient.

Cu as an inhibitor of the succinic dehydrogenase

It is well known that Cu reacts with SH groups and if the activity of the dehydrogenase calls for the integrity of such groups in its structure, it is to be expected that its presence will inhibit activity. This is the case. Quastel & Wooldridge [1927] found that treatment with $CuSO_4$ inhibited the succinic enzyme as well as some other dehydrogenases in the case of *B. coli* but we have found no reference to the effects of the metal on the enzyme of animal tissues.

Exp. 4. To equal samples of washed muscle, treated as usual and suspended in buffer solution, $CuSO_4$ was added to give the concentrations of Cu shown in the following table. The reduction times were determined immediately after the addition.

Exp. 4

Cu added μg.	Reduction times min.
0	10
5	11
10	12
40	15
100	80

It will be seen that with the amount of tissue used in this as in other experiments (0·5 g.) the metal has relatively little effect on the enzyme up to a concentration of 40μg. in 3 ml. solution. With 100μg. it becomes large.

These results bear on those of the earlier experiments of Hopkins and Morgan and on some of our own. By far the most convenient method for preparing GSSG from GSH is that of Pirie [1931] in which the latter is oxidized by H_2O_2 in the presence of small amounts of Cu. The oxidized product so made which has been chiefly used in this laboratory contains traces of Cu. This was ignored in the previous paper chiefly because it was found that cystine and cysteine absolutely free from Cu could in essentials replace glutathione in experiments described. It seemed to us desirable nevertheless to study the effect of these small amounts of the metal. The amount of Cu present in the preparations of GSSG was determined by the method of Callan & Henderson as developed by Haddock & Evers [1932] and found to be 10μg. in 100 mg. (1 in 10,000). The presence of this amount as shown by experiments described below is not without effect on the apparent rate of enzyme inactivation by GSSG. It is sure however that the action of glutathione and that of the metal are distinct in kind. We have found that inactivation of the enzyme by GSSG occurs typically in the complete absence of Cu (Exp. 8).

Exp. 5 again illustrates the effect on the enzyme activity of small amounts (10μg.) and of larger amounts (100μg.) of Cu, and shows that in the case of the smaller amounts incubation does not increase the effect of the metal. This is exerted immediately. Of the tissue 0·5 g. was in 3 ml. buffer solution as usual. With each concentration of metal the reduction time was determined on one sample immediately after adding the metal. Two other samples in each case were incubated in its presence for two successive periods of 3 hr., the tissue being washed in between. One of the latter samples was then incubated with $M/10$ GSH solution. All reductions with succinate as substrate.

Exp. 5

	Reduction times
No Cu	
Control untreated	12 min.
10 μg. Cu	
Determined immediately	14 min.
Incubated	13 min.
Incubated, then GSH	12 min.
100 μg. Cu	
Determined immediately	45 min.
Incubated	5 hr.
Incubated, then GSH	17 min.

The effect of 10μg. Cu, the amount contained in 0·1 g. of the GSSG preparation described above (and therefore the amount usually present in our experiments with that preparation), though small, has been always observed and was of the same order in other experiments. It is noteworthy that with ten times this amount of metal, though marked diminution of the enzyme activity occurs at once, the effect increases with time. This circumstance will receive later reference. Subsequent exposure to the influence of GSH always restores the activity of the Cu-treated enzyme though usually not quite completely.

Experiments were carried out to determine to what extent the small amount of Cu in GSSG preparations prepared as described above is concerned in the inactivation observed when enzyme preparations are exposed to them.

Exp. 6. Dithiodiethyl carbamate is well known to form a complex with Cu. The following experiment was made to show (1) whether the carbamate has by itself any effect on the enzyme, (2) how far it prevents the effect of added Cu on its activity and (3) to what extent its presence affects the rate at which the activity

disappears when enzyme preparations are exposed to solutions of GSSG. 1 g. Latapie-minced washed muscle was incubated for 3 hr. in solutions at *p*H 7·4 containing the various additions mentioned. 2 ml. 0·1% carbamate were employed and in each case the total volume was 5 ml. After incubation the tissue samples were filtered off, washed and the reduction times determined as usual.

Exp. 6

	Reduction times
Control (muscle alone)	11 min.
With carbamate only	11 min.
With 10 μg. Cu	14½ min.
+ carbamate	11 min.
With GSSG (0·1 g. = 10 μg. Cu)	5 hr.
+ carbamate	3 hr. 50 min.

It will be seen that the carbamate had by itself no influence on the enzyme, while it protected it from the inhibitory effect of the added Cu. Its presence reduced the reduction time of tissue incubated with GSSG from 300 min. to 220 min. The effect of the metal seems to be in some sense additive.

It was felt desirable to add to the evidence provided by carbamate inhibition by using, if possible, GSSG preparations wholly free from Cu. We have found that if preparations containing such amounts of the metal as those made by Pirie's method are shaken in solution with an adequate amount of freshly washed minced muscle, the Cu is completely removed by adsorption on the tissue, or reduced to amounts too small to be demonstrated.

Exp. 7 was carried out with GSSG thus freed from Cu, and its effect on the enzyme compared with that of an equal concentration of the original preparation.

Exp. 7

	Reduction times
Muscle incubated without GSSG	5 min. 20 sec.
Incubated with the original preparation of GSSG	72 min.
Incubated with Cu-free GSSG	56 min.

The reduction time is again larger in the presence of the Cu.

In Exps. 6 and 7 the tissue was exposed only once to the GSSG solution. This, as has been frequently pointed out, is never adequate, at least with reasonable concentrations, to remove the enzyme activity completely.[1]

We wished therefore to show whether with adequate exposure the activity could be completely removed by a Cu-free preparation. This was the case, or nearly the case, in Exp. 8. The copper was removed by shaking 30 ml. *N*/10 GSSG at *p*H 7·4 with 2·25 g. of well-washed muscle. The tissue was filtered off after 5 min. Two samples of 1 g. tissue, prepared as usual, were incubated for two successive periods of 3 hr. each with 5 ml. *M*/10 Cu-free GSSG. A third equal sample was similarly treated but with the original GSSG preparation, and a fourth in the presence of 10 μg. Cu alone. All samples were prepared for the reduction time determinations in the customary way.

The Cu-free preparation reduced somewhat more rapidly than the original, but the difference was slight. The experiment showed clearly that contact with

[1] The plan and extent of our experiments have been sometimes limited by the available supply of glutathione. Over 50 g. have been used in this research. All has been prepared in this laboratory.

GSSG when free from the metal can completely remove the activity of the dehydrogenase.

Exp. 8

	Reduction times
Control (muscle incubated alone)	10 min.
Incubated with Cu-free GSSG	8 hr. +
+ carbamate	8 hr. +
Incubated with original GSSG	8 hr. + +
Incubated with 10 μg. Cu	14 min.

Other inhibitors influencing SH groups

Morgan & Friedmann [1938, 1] having shown that maleic acid by reacting with SH groups forms stable addition compounds with thiolacetic acid, cysteine and glutathione, showed further [1938, 2] that it reacts with the fixed SH groups of proteins and therefore inhibits such enzymic activities as call for the integrity of these groups. Hopkins & Morgan [1938] having shown that the activity of the succinic dehydrogenase comes into this category, the first-mentioned investigators were led to study the effect of maleic acid upon it, finding that well-marked inhibition occurs [Morgan & Friedmann, 1938, 2]. They had previously found however that interactions of thiols with the acid reach equilibria and do not proceed to completion. The following experiments confirm and extend their observations in the case of the succinic enzyme.

Exp. 9 involved a comparison between maleic and fumaric acids. Of a washed muscle preparation 3 equal portions were incubated overnight, alone in phosphate buffer (control), in $M/10$ maleic acid and in $M/10$ fumaric acid respectively, all at pH 7·4. Each portion was then submitted to the same treatment for a second time. They were then well washed at the centrifuge and their reduction times in the presence of succinate determined.

Exp. 9

	Reduction times (min.)	
	Once incubated	Twice incubated
Control	12	12
Maleic acid	27	53
Fumaric acid	13	12

It is seen that the inhibition by maleic acid is marked but involves a slow reaction not going to completion. Fumaric acid has no effect upon the enzyme.

Rapkine [1933] has shown that iodoacetic acid reacts with the SH group of proteins, and its influence as an inhibitor of the dehydrogenase was discussed by Hopkins & Morgan [1938]. We have confirmed their results in finding that its action on the enzyme is a relatively slow process, increasing at any given concentration with the time of incubation. We have ourselves found that its effects are irreversible, not removed by washing the enzyme preparation or under the influence of GSH. In one experiment for instance involving the customary technique we found that an untreated preparation with a reduction time of 12 min. gave, after incubation with $M/25$ iodoacetic acid for 1 hr., one of 120 min.; and when afterwards incubated with GSH, 130 min. In another experiment, in which the control time was $12\frac{1}{2}$ min., the preparation after exposure to iodoacetic acid required 91 min. although it was first very thoroughly washed. The effects are strictly irreversible.

Protection of the dehydrogenase from the influence of GSSG

Malonic acid is well known as an inhibitor of the activity of succinic dehydrogenase and must therefore enter into some effective association with its active structure. We therefore investigated its ability to protect the enzyme from oxidation by GSSG. In experiments for this purpose it is important to know that malonate can be easily removed from its association with the enzyme by washing the tissue preparations which have been exposed to solutions containing it. Unless the concentrations are high this is the case after relatively few washings as shown by the following experiments. Two 0·5 g. portions of a muscle preparation were placed in 2 ml. $M/10$ malonic acid. They were incubated anaerobically for $\frac{1}{2}$ hr., filtered off, and, in one case, the tissue was washed thrice on a linen filter before the reduction times were determined and in the other left unwashed. In the latter case the presence of the malonic acid on the surface naturally inhibited the enzyme. The control was an equal portion of the tissue incubated in buffer solution alone. The following reduction times were obtained. Control, 15 min.; treated with malonic acid and not washed, 61 min.; washed sample, 17 min. It is seen that the washed sample was nearly as active as the original. Although in some experiments we have first incubated the enzyme preparation with the malonic acid its association with the enzyme is in fact established immediately (Exp. 11).

The following experiments show that malonic acid can very completely protect the enzyme from oxidation by GSSG.

Exp. 10 shows that protection occurs with very low concentrations. Latapie-minced washed muscle in portions of 0·5 g. was incubated for 2 hr. under the usual conditions with $M/10$ GSSG, respectively unprotected and protected by the presence of malonic acid in various concentrations. The following reduction times were obtained.

Exp. 10

	Reduction times
Original tissue untreated	9 min.
Incubated with GSSG unprotected	2 hr. 30 min.
with malonate $M/10$	15 min.
,, $M/50$	10 min.
,, $M/100$	11 min.
,, $M/500$	11 min.
,, $M/1000$	13 min.
,, $M/5000$	17 min.

It is clear that a large degree of protection is afforded by very low concentrations of malonate and that the effect decreases only slowly with a fall in these. It is probable that in the above experiment the tissue from the strongest ($M/10$) solution was not quite adequately washed and the small amount of malonic acid still adhering somewhat increased the reduction time.

Exp. 11 carried out with the same tissue preparation as in Exp. 10 shows that the formation of the association between malonic acid and the enzyme is a rapid process scarcely affected by a time factor. The tissue in $M/50$ malonic acid was incubated in Thunberg tubes with hollow turn-over stoppers containing $M/10$ GSSG. The latter was emptied into the tubes at successive intervals during incubation which in each case was afterwards continued for 2 hr. Tissue samples were filtered off and washed free from malonic acid before the reduction experiments were carried out.

Exp. 11

GSSG solution added	Reduction times min.	sec.
At once	13	30
After 5 min.	12	30
After 10 min.	14	45
After 30 min.	14	40

Previous incubation is seen to have but little effect on the degree of protection from the influence of the GSSG. As shown in the protocol of Exp. 10 the reduction time of this preparation after exposure to the GSSG solution alone for 2 hr. unprotected was 2 hr. 30 min.

The following experiments illustrate further the efficiency of malonic acid in protecting the enzyme from the influence of GSSG.

Exp. 12. In this equal samples of a muscle preparation were incubated for two successive periods of 3 hr. each respectively in $M/10$ GSSG alone and in the same with $M/10$ malonic acid present. A control sample was, as in all experiments, incubated for similar periods in the buffer solution alone. After washing the following reduction times were obtained:

Exp. 12

	Reduction times
Control	5 min. 45 sec.
Incubated with GSSG alone	5 hr. +
In presence of malonate	8 min.

Exp. 13. In this the muscle tissue used was passed twice through the Latapie mincer with the intention of increasing its permeability. The experiment was otherwise essentially the same in plan as the foregoing but, in the incubations, while the concentration of GSSG was the same, that of the malonic acid was only $M/50$. The incubations were two, of 4 hr. each. The tissues were very thoroughly washed before the reduction times were compared.

Exp. 13

	Reduction times
Control	8 min.
Incubated with GSSG alone	5 hr. 30 min.
In presence of malonate	9 min. 30 sec.

Of *Exp. 13a* it need only be said that the procedure was similar, and the muscle preparation the same as in Exp. 13, but the incubation times were shorter and the enzyme therefore not completely inactivated by GSSG. The reduction times were as follow: control, 9 min.; after GSSG alone, 4 hr.; after GSSG plus malonate, 9 min.

The influence of substances, other than malonic acid, capable of protecting the succinic enzyme from the influence of GSSG was studied. Other dibasic acids displayed this property more definitely than other substances tried, among them, as might be expected, succinic acid itself, but also fumaric and malic; pyrophosphoric protects though generally less efficiently than the others.

The results of *Exp. 14* demonstrate such effects. A muscle preparation was incubated for 4 hr. with GSSG solution alone, and also in the presence of the substances shown. All samples were thoroughly washed.

Exp. 14

	Reduction times
GSSG alone	3 hr. +
With succinate *M*/10	4 min.
With fumarate *M*/10	3 min.
With malonate *M*/10	4 min.
With pyrophosphate *M*/50	4 min.

In this experiment, in which the enzyme preparation was exceptionally active, protection by all four substances was very complete. Other experiments have confirmed these results though, as stated, pyrophosphate has usually proved somewhat less efficient than the others.

It seemed worth while to try the effect of a greater variety of possible protectors. In the following (Exp. 15) the GSSG and the other substances were all employed in *M*/10 solution. The incubation period was 4 hr.

Exp. 15

	Reduction times
Original tissue (untreated)	10 min.
GSSG alone	3 hr.
With addition of:	
Acetate	4 hr. +
Phenylacetate	4 hr. +
Malonate	13 min.
Hydroxymalonate	20 min.
Lactate	2 hr.
Glucose	1 hr.
Sucrose	2 hr.

The monobasic acids other than lactic afford no protection. The reason for the definite but relatively smaller protection by lactic acid and glucose lacks explanation. Hydroxymalonic is nearly as efficient as malonic acid itself. Except for these two, none of the substances tried is an inhibitor of the enzymic activity.

Various authors have shown that the most efficient inhibitors of the succinic dehydrogenase activity are dicarboxylic acids [Quastel & Wooldridge, 1928; Potter & Elvehjem, 1937; Leloir & Dixon, 1937]. Two acidic groups seem to enter into special relations with the enzyme. It was to be expected that inhibitors will also protect the enzyme from oxidation.

Protection from the influence of GSH

It was desirable to discover whether substances which protect from oxidation by GSSG also protect from subsequent reduction by GSH. In the following experiment a muscle preparation was first incubated twice with GSSG, and the succinic enzyme present proved to be completely inactivated. The tissue was then divided into portions of 0·6 g. each, and these were incubated, one sample with GSH alone, and others with the substances indicated below. The GSH was in *M*/10 solution, buffered as usual. It was placed in the hollow stoppers of Thunberg tubes and added after the samples of oxidized tissue had been incubated anaerobically for ½ hr. with the materials under study. The samples were then washed and their reduction times determined.

Succinic and fumaric acids inhibit the reduction to some extent; the others not at all. That malonic acid protects from GSSG so completely and from GSH not at all suggests structural relations which may prove instructive.

Exp. 16

	Reduction times min.
Control (tissue untreated)	12
Oxidized by GSSG	∞
Re-reduced by GSH alone	12
GSH + succinic acid	19½
+ malonic acid	13
+ fumaric acid	17
+ pyrophosphoric acid	12½

Protection of the enzyme thiol groups from the influence of GSSG

When the nitroprusside test is used to compare the concentration of SH groups in active and inactivated preparations respectively it should be realized that a very low intensity in the test is compatible with activity.

If SH groups are necessary for the activity of the succinic dehydrogenase, and for this there seems to be strong and cumulative evidence, it is clear that malonic acid and other protectors of the enzyme activity must prevent that group from being oxidized by GSSG. It is by no means easy to prove directly that this is the case, nor is it perhaps easy to picture a mechanism for such protection. Malonic acid, for instance, apparently establishes no special relations with thiol groups, so that the protection must in any case be indirect.

So long as we remain unable to isolate the enzyme, or obtain preparations in which it is present in higher relative concentrations than in whole tissues merely washed, it is somewhat difficult to employ the nitroprusside reaction in proof that the SH groups proper to the enzyme itself have or have not been protected. They must represent but a minute portion of the thiol groups present in the tissue as a whole. That the active surface of the specific enzyme compared with the surfaces presented by disintegrated muscle must be exceedingly small cannot be doubted, and this seems to be illustrated by the minute concentration in which malonic acid can protect the enzyme (Exp. 10).

If the nitroprusside reaction given by a tissue sample which has been exposed unprotected to GSSG be compared with that given by a corresponding sample protected by malonic acid, though the activity of the enzyme is nearly lost in the former and intact in the latter, the reaction may be slight in both cases and a difficulty may be felt in deciding whether there is a significant difference between them. It is however easier to distinguish if the two samples be extracted with trichloroacetic acid and the test applied to the solution. It can be said definitely that in our experiments, though the difference may have been slight in some cases, it has always been more intense in a protected sample. It seemed desirable however to obtain some degree of quantitative evidence for this, and we have succeeded in doing so by incubating anaerobically with solutions of GSSG the samples to be compared. These were then titrated with very weak standard I_2 in the presence of KI in order to determine the amount of GSH produced by the interaction between the protein SH groups in the tissue preparation and the GSSG in solution. The quantities involved are doubtless too small for accuracy, but they allow of comparisons which are significant.

In each of two experiments sufficient of a preparation of muscle was made to carry out the above treatment, as well as determinations of enzyme activity. It was incubated with GSSG anaerobically in large Thunberg tubes for adequate periods, with and without the presence of malonate. The respective reduction times showed, in each experiment, that in the absence of protection the enzyme activity had nearly disappeared, somewhat more completely in the first experi-

ment than in the second. It was fully preserved in the presence of malonate. After incubation as above the evacuated tubes were opened under a solution of trichloroacetic acid, the tissue was filtered off and the filtrates titrated with $N/5000$ I_2. The end points could be observed without difficulty. The following I_2 equivalents are calculated in each case for 5 g. tissue preparation.

	ml. $N/5000$ iodine	
	Exp. 1	Exp. 2
Incubated with GSSG alone	1·2	7·8
Incubated with malonate present	16·0	19·5

There seems to be no doubt that the enzyme SH groups are protected by malonic acid in some specific way.

We may add that in an occasional experiment when treatment of the tissue with GSSG had made the nitroprusside reaction completely negative the enzyme was found to be inactive. This result is relatively easy to obtain, as Hopkins & Morgan [1938] found, when heart muscle is the source of the enzyme. With the less permeable preparations from skeletal muscle the treatment must be prolonged and repeated. That the enzyme structure nevertheless remains intact save for the disappearance of the SH groups may be shown by subsequent treatment with GSH when the activity is fully restored.

Oxidation of SH groups by alloxan

It was shown by Purr [1935] that papain and cathepsin are inactivated by alloxan owing to the fact that it oxidizes SH groups in the structure of these enzymes. We have tested its effect on succinic dehydrogenase and find that in adequate concentration it inactivates it, the effect being reversible (though apparently not completely) under the influence of GSH. The presence of succinic acid protects from oxidation by alloxan, but malonic gives relatively very little protection. The following experiments illustrate these statements. It should be here remarked that the nitroprusside test shows that alloxan oxidizes the SH groups of a muscle preparation very quickly, even in low concentrations ($M/100$, $M/50$). When used in $M/10$ solution it gives the familiar pink colour with the muscle protein which has been recently studied by Lieben & Edel [1932].

In the following experiment a muscle preparation, made as usual, was incubated for 30 min. with $M/100$ and $M/50$ alloxan. Samples were well washed and their reduction times determined. The activity of the α-glycerophosphate enzyme was determined at the same time, so it is convenient to give the results here. Other samples of the oxidized material were washed and then incubated for $1\frac{1}{2}$ hr. with $M/10$ GSH, again washed thoroughly and submitted to reduction tests. In the protocol SD = the succinic and GLD = the glycerophosphoric enzyme.

Exp. 17

		Reduction times min.
Control untreated	SD	15
	GLD	8
Alloxan $M/50$	SD	50
	GLD	8
Alloxan $M/100$	SD	56
	GLD	8
GSH after alloxan $M/50$	SD	20
	GLD	8
GSH after alloxan $M/100$	SD	22
	GLD	8

The glycerophosphoric enzyme is seen to be unaffected by alloxan at this concentration. GSH reverses its effect upon the succinic enzyme. Other experiments have given similar results. The following shows that the alloxan reaction is progressive with time. The preliminary treatment of the samples was the same as in the last case.

Exp. 17a

	Control	*M*/50 alloxan filtered off immediately	*M*/50 alloxan incubated 30 min.
Reduction times (min.)	SD 11	15	60
	GLD $4\frac{1}{2}$	$4\frac{1}{2}$	$4\frac{1}{2}$

Finally the following shows that as stated above the protection of the enzyme by succinic and malonic acids from oxidation by alloxan differs in an interesting way from the protection they afford from oxidation by GSSG. Succinic acid affords a considerable degree of protection, malonic very little.

	Reduction times (min.)
Control	10
Alloxan alone	35
Alloxan + malonate	26
Alloxan + succinate	14

Alloxan in concentrations of the order of *M*/10 has effects on the enzyme which must be additional to the oxidation of thiol groups, and when incubated with it all the dehydrogenases we have studied lose much of their activity.

II. Other dehydrogenases

We have conducted experiments, precisely similar in kind to those of the previous section, with the α-glycerophosphate, lactic and malic dehydrogenases; also with the aldehyde mutase and alcohol dehydrogenase of horse liver (prepared by the method of Dixon & Lutwak-Mann [1937]) and with two enzymes of the oxidase type, namely the cytochrome (indophenol) oxidase and the xanthine oxidase. None of these enzymes is appreciably influenced by glutathione. Consistent with this is the circumstance that their activity, except for that of the liver aldehyde mutase, is not affected, or affected to a small extent only, by inhibitors which are believed to act by suppressing the influence of thiol groups.

The experiments with the succinic dehydrogenase have been described in detail and those of the present section were essentially similar in all respects. The evidence can therefore be more briefly presented. To the lactic and malic enzymes cozymase was of course added when the reduction times were determined.

Effects of incubation with GSSG *and* GSH

In the following table the results of several experiments are collected.

	Reduction times (min.)			
Enzyme	Original	GSSG alone	GSSG with malonate	GSH
Glycerophosphate	6	$6\frac{1}{2}$	6	6
,,	10	10	10	10
Lactic	3	5	—	—
,,	$3\frac{1}{2}$	5	$4\frac{1}{2}$	$5\frac{1}{2}$
,,	$4\frac{1}{2}$	7	7	—
Lactic (from horse liver)	20	21	—	22
Malic	$6\frac{1}{2}$	$8\frac{1}{2}$	—	—
,,	9	13	—	—

In all cases the influence of GSSG was absent or negligible.

Effect of Cu

The relatively small effect of Cu on the lactic dehydrogenase is illustrated in the following experiment. The preparation was not incubated with the metal but the effect was tested immediately after its addition.

	Reduction times min.
Control (+cozymase and lactate)	$3\frac{1}{4}$
With 5 μg. Cu	4
With 50 μg. Cu	6
With 5 μg. Cu (+dithiodiethylcarbamate)	$3\frac{1}{2}$
With 50 μg. Cu (+dithiodiethylcarbamate)	$3\frac{3}{4}$

In the cases of the lactic and malic enzymes the differences between the effect of incubation with the GSSG preparation containing 10 μg. Cu in 100 mg. of GSSG (see previous section) and that with the Cu-free preparation were compared in exactly similar circumstances. The results should be compared with Exp. 7 in which the same tissue was employed.

	Reduction time (min.)	
	With lactate	With malate
Tissue incubated alone	$2\frac{1}{2}$	6
With GSSG containing Cu	$5\frac{1}{2}$	8
With GSSG Cu-free	$2\frac{3}{4}$	$7\frac{1}{4}$

In one experiment with the glycerophosphate enzyme 10 μg. Cu were added to two equal portions of a tissue preparation, treated otherwise as usual. In one case the reduction time was taken immediately; in the other the tissue was first incubated with the metal for 3 hr. The control was reduced in 7 min.; the sample with Cu, unincubated, 8 min.; the incubated sample 10 min.

Effects of maleic and iodoacetic acids

The following experiments illustrate the absence of appreciable inhibition due to maleic (and fumaric) acids. The results were confirmed in other cases.

	Reduction times (min.)		
Enzyme	Controls	Maleic acid	Fumaric acid
Lactic	3	$3\frac{1}{2}$	3
Glycerophosphate	4	4	4
Malic	$14\frac{1}{2}$	$15\frac{1}{2}$	$14\frac{1}{2}$

The following show that the same is true of iodoacetic acid even after incubation with $M/50$ solution for 1 hr.

	Reduction times (min.)	
Enzyme	Control	Incubated with I.A.
Lactic	$3\frac{1}{2}$	4
,,	$3\frac{1}{4}$	$3\frac{1}{2}$
Glycerophosphate	7	10

In this group of dehydrogenases, as will be seen from the various protocols given in this section, the influence of glutathione is absent, or, in comparison with the case of the succinic enzyme, negligible. The glycerophosphate enzyme has always shown remarkable resistance to the effects of the treatment involved in our experiments, its reduction times remaining constant at all stages; the lactic and malic enzymes have proved somewhat less stable but it will be seen

that their resistance to glutathione is definite enough. Consistent with this, in proof that thiol groups are not essential to their activity, is the circumstance that these enzymes are much less affected by Cu than is the succinic enzyme, and, unlike it, are not affected by the irreversible effects of maleic and iodoacetic acids.

The influence of glutathione on two other enzymes was tested, namely the aldehyde mutase and the alcohol dehydrogenase from horse liver. In neither case did exposure to GSSG affect the activity though the mutase has been said to be sensitive to iodoacetic acid [Dixon & Lutwak-Mann, 1937].

Oxidases

Experiments made with the Cu-containing preparation of GSSG described in the previous section led us at first to believe that the xanthine "oxidase" resembled the succinic enzyme in its relations with glutathione. This however proved to be due to its great sensitiveness to Cu, a property which had been previously recorded by others [cf. Andersson, 1936]. Small amounts of Cu equivalent to that contained in the glutathione removed the enzyme activity to an equal degree. The effect of Cu is however reversible and completely removed by treatment with GSH. It would seem that this enzyme may prove not to be a typical oxidase, but at the moment it may be left with the title.

The cytochrome (indophenol) oxidase was also studied. Its activity was measured by using phenylenediamine as a substrate and also by testing spectroscopically its ability to oxidize cytochrome. Its activity was found to be entirely resistant to the influence of GSSG.

Cytochrome-reduction method for determining enzyme activity

It is of interest in the case of those dehydrogenases which reduce the pigment to observe with a spectroscope the rate at which the prominent bands of reduced cytochrome *c* appear when the oxidized form is added to a solution containing

Enzyme preparations treated with following substances	The spectrum of reduced cytochrome *c* on adding the substrate became: Just visible after			Fully developed after		
	SD	GLD	LD	SD	GLD	LD
Control untreated	At once	At once	At once	50 sec.	20 sec.	25 sec.
GSSG	No bands at any time	40 sec.	50 sec.	No bands	1 min.	3 min.
,, then GSH	At once	At once	1 min.	20 sec.	20 sec.	2 min.
,, Cu-free	No bands at any time	—	—	No bands	30 sec.	1 min.
,, +malonate	2 min.	—	—	4 min.	—	—
,, +succinate	5 min.	—	—	7 min.	—	—
,, +pyrophosphate	8 min.	—	—	13 min.	—	—
Cu high concentration	40 min.	—	5 min.	Several hours	—	10 min.
Cu low concentration	2 min.	1 min.	2 min.	4 min.	2 min.	5 min.
Alloxan *M*/50	60 min.	1 min.	—	2 hr.	3 min.	—
Iodoacetic acid *M*/80	22 min.	2 min.	2 min.	1 hr.	3 min.	15 min.
Iodoacetic* acid (muscle *in vivo*)	1 hr.	—	—	Several hours	—	—
Maleic acid	17 min.	—	—	30 min.	—	—

* In this experiment iodoacetic acid was injected into the lymph sac of frogs. One hind limb was removed before the injection as a control, and the motor nerves to the muscles of the other limb were cut. When rigor was evident in the forelimbs the second limb was removed. The muscles in each case were then ground up with a little water and washed till free from haemoglobin, and the succinic dehydrogenase activity determined spectroscopically. In the control the cytochrome bands were fully established in 30 min.

an enzyme and its specific substrate. It forms a check on the methylene blue results and is a convenient and rapid method for deciding whether an enzyme is present or absent, giving also approximate information as to the degree of its activity. It will be seen on examining the above table that results obtained with the methylene blue method are confirmed in every instance. The method of observation was of the simplest. A test tube containing the enzyme preparation, suspended in phosphate buffer at *p*H 7·4 together with a moderate concentration of oxidized cytochrome *c*, was clamped before the slit of a fixed straight vision spectroscope with an adequate light source.[1] A stopwatch was used and the times taken from the moment of adding the substrate (1) to the first observable appearance of the bands, and (2) to the establishment of their maximum intensity. The descriptions given in column 1 of the table will be understood from what has gone before.

The following is an experiment in which was measured the O_2 uptake by systems containing tissue preparations which had been previously treated with GSSG, unprotected and protected. One sample after oxidation was re-reduced with GSH. The substrate was succinate, and cytochrome *c* was added to the system. The necessary cytochrome oxidase was present in sufficient concentration in the muscle preparations. Barcroft manometers: O_2 uptakes are given in μl. per hr.: reduction times for the corresponding samples.

Previous treatment of tissue	O_2 uptake	Reduction times
Original	280	11 min.
Incubated with:		
GSSG alone	0	7 hr. +
,, with malonate	210	14 min.
,, with succinate	190	19 min.
,, with pyrophosphate	140	16 min.
,, then GSG	310	10 min.

It will be seen that as measures of enzyme activity the two methods are in close correspondence.

Addendum

Location of the dedydrogenase in muscle

We have found that if muscle tissue minced with the Latapie is thoroughly extracted with salt solutions as for the extraction of myosin etc., the extraction being continued until no trace of protein is present in a final extract, the fragmented fibres, of which the majority when examined under the microscope are seen to be severed transversely, still show cross striation and—as we have repeatedly observed—typical birefringence of the discs. That cross striation remained intact after continued extraction with NH_4Cl was observed by Danelewsky [1882]. That author believed—perhaps on not very convincing evidence—that a relatively high proportion of this "framework" in their fibres was characteristic of muscles with the greatest functional activity. We have found that muscle tissue after exhaustive extraction with salts still contains succinic, α-glycerophosphoric, lactic and malic dehydrogenases in relatively high concentrations.

The saline extracts, unless they obviously contain dispersed colloidal particles, are found to display no dehydrogenase activity. The later extracts of a series of extractions can be obtained, after centrifuging, perfectly clear, and these are quite free from these enzymes though in the extracted residue their concentrations are high. If the completely extracted tissue be now treated with, say, 10 times its volume of *N*/100 HCl, more protein is extracted and the final residue

[1] The experiments with the lactic dehydrogenase were carried out anaerobically.

becomes gelatinous. It can however be centrifuged off in a high speed centrifuge and is then found to be inactive, as is also the protein extracted by the acid. The framework structure of the fibres immediately disappears on treatment with HCl.

It is true, to judge from methylene blue reductions, that the concentrations of the enzymes when compared with those in the original tissue are not increased proportionately with the removal of so large a proportion of the muscle substance (some 85% of the total muscle proteins), so that what happens during the prolonged treatment is not entirely clear. The facts stated may be illustrated by the following experiment, typical of several made.

Latapie-minced muscle (25 g.) was first extracted four times with 200 ml. NaCl (10%) and centrifuged after each extraction; the second extract stood overnight in the refrigerator. It was then extracted thrice with LiCl [Bate Smith, 1935; 1937]. The last extract contained no trace of protein. The final residue in which the fibres showed typical cross striation was washed and then centrifuged until freed as far as possible from water. Equal weights (0·5 g.) were used in determining the following reduction times, but the equivalence of this weight with that of the original minced muscle was not accurately known.

	Reduction times		
Dehydrogenase	Control	Incubated GSSG	Re-incubated GSH
Succinic	12 min. 30 sec.	5 hr.	10 min.
α-Glycerophosphate	6 min.	6 min.	6 min.
Lactic	5 min. 30 sec.	11 min.	—
Malic	8 min. 40 sec.	13 min.	—

It will be seen that the enzymes in the extracted tissue show the same relations as those always observed with tissue when simply washed with water.

In a special experiment in every way similar to this the enzyme activity of the saline extracts was examined. The order of the extractions was the same as in the above. A sample of the fourth NaCl extract and one of the first LiCl extract were dialysed and in each case the precipitate which separated was centrifuged off, washed and tested for enzyme activity. The precipitate from the NaCl extract contained none of the succinic but very small amounts of the glycerophosphate enzyme and doubtful traces of the others. That from the LiCl extract was practically free from any activity. All the enzymes were present in the residue in concentrations comparable with those above. The supernatant solutions in the dialyser were free from activity.

It seems clear from the recent work of Weber [1934], Edsall & von Muralt [1930], Bate Smith [1935] and others that the protein which resists salt-extraction is mainly myosin itself, protected in some way from removal. In the case of Latapie-minced muscle it seems unlikely that this is due to the impermeability of the membranes, as the fibres are almost all broken across. Bate Smith [1937], who confirmed the presence in muscle of "globulin x" a protein first described by Meyer & Weber, is of opinion that some of this is associated with myosin in the residue after salt extraction (personal communication). It would be of interest, as he suggests, if, while myosin molecules form the basis of the contractile substances as is now generally admitted, the enzymes were associated with the above globulin which is in close relation with the myosin.

The circumstance that during and after the salt extraction the dehydrogenases seem to remain confined to the residual structural framework, while the degree of consequent increase in their concentration is not what would be

expected, calls for explanation. It is conceivable that denaturation changes during the extractions may be in part responsible for this.

In any case it is of interest to know that enzymes controlling energy-yielding reactions exist in this close relation with the structural framework of the fibres.

Discussion

The experiments in this paper have fully confirmed those of Hopkins & Morgan in showing that the activity of the succinic dehydrogenase is completely removed when preparations containing it are incubated with GSSG and fully restored on subsequent incubation with GSH. Those authors showed that, in contrast, the α-glycerophosphate dehydrogenase is wholly unaffected by this treatment. We have extended the list of resistant enzymes by the inclusion of the lactic and malic dehydrogenases, the alcohol dehydrogenase and the aldehyde mutase from horse liver, xanthine oxidase and cytochrome oxidase. Rapkine [1938] has recently shown that the triosephosphate mutase from muscle may be inactivated by GSSG; but, to judge from our results, the succinic enzyme seems likely to prove the only typical dehydrogenase which undergoes this inactivation, and therefore probably the only one which requires for its activity the integrity of the SH groups in its specific protein structure. We have added to the evidence for the importance of such groups in the case of that enzyme by showing that Cu, maleic acid and iodoacetic acid, which are known or believed to react with thiol groups, are inhibitors of the enzyme, while having little or no effect on the activity of the other dehydrogenases tested. Of the above, Cu inhibits reversibly; the two acids irreversibly.

Nearly all our experiments have been made on skeletal muscle tissue, but Hopkins & Morgan showed that, so far at least as the effects of glutathione are concerned, preparations of the enzyme from cardiac muscle, kidney and liver behave identically. The evidence that these effects are due to oxidation and re-reduction of thiol groups is in any case strong, but we have perhaps added to it by showing that the inactivation by GSSG involves a progressive chemical reaction with a somewhat high temperature coefficient (Exps. 1, 2, and 3). If the inactivation were due merely to the formation of some reversible association of GSSG with the enzyme in which the latter is inactive but which dissociates under the influence of GSH (this being perhaps possible though unlikely) its kinetics would not be of this kind.

Alloxan, as an oxidant of SH groups, when in relatively low concentrations ($M/50$, $M/100$) inactivates the succinic dehydrogenase. In contrast, at such concentrations it has no effect at all on the α-glycerophosphate enzyme (Exp. 17). At high concentrations ($M/10$), when it produces the characteristic pink colour with the tissue proteins, it exercises a less specific destructive effect on all the enzymes we have tested. If the concentrations used are of the lower order its effects on the succinic enzyme are reversed by reduction with GSH; otherwise the reactivation is only partial.

Cu is well known to react with thiol groups and it was to be expected that an enzyme of which the activity calls for the integrity of such groups in its structure would be inhibited by the presence of the metal. It proved easy to show that this is true of the succinic dehydrogenase. With adequate Cu concentrations the inhibition is complete and immediate. With tissue preparations however, such as have been generally used by others and by ourselves in studies of this enzyme, certain difficulties arise in points of detail, and these were evident when the effects of varying the concentration of Cu were being studied.

Since Thunberg [1909] first demonstrated the existence of this dehydrogenase most experiments made with it, as well as those with other "insoluble" dehydrogenases, have involved the use of whole tissues which were merely minced (or sometimes more completely disintegrated) and then thoroughly extracted with water. In any such preparation the specific protein of any one enzyme is in association with those of other enzymes, and, of course, also with a relatively very large proportion of indifferent tissue proteins. In numerous experiments this circumstance has proved to be of no consequence, as the literature of the subject shows. When, however, the significance of some detail in the specific enzyme protein is to be determined difficulties may intrude. When for instance Cu is added to such a tissue preparation it must be expected to react with the SH groups of the indifferent tissue proteins present no less than with those proper to the enzyme, and, of course, the number of the former present must be relatively very great. Only a small fraction of the metal added may therefore be effective. An added amount which, were it possible to work with the enzyme in a concentrated form, might produce strong inhibition may with such tissue preparations have little effect. This was the case with the lower concentrations of Cu employed in our experiments (10–20 μg. per g. tissue). Inhibition even with these was always to be observed, but it was slight.

With adequate concentrations of Cu, which are relatively large (in the conditions of our experiments 300 μg. for each g. muscle tissue), inhibition, as already stated, is immediate and complete. With intermediate concentrations (e.g. 100 μg.) it might seem from Exp. 5 that a time factor is involved in the process. This however is unlikely and it would seem rather that the nature of the enzyme preparation is again involved, and that a permeability factor intervenes. While with such concentrations the amount of Cu reaching the dehydrogenase itself may be adequate to induce noteworthy inhibition, it yet takes an appreciable time after its addition to the washed tissue to reach the enzyme surface.

These various relations seem to call for comment, especially as they bear in a general way on the customary use of such tissue preparations. They do not however obscure the proof that the activity of the dehydrogenase can be completely inhibited by Cu. On the other hand our experiments have shown that the lactic, malic and α-glycerophosphate dehydrogenases, in keeping with their resistance to oxidation by GSSG, are affected to a very much smaller extent by the presence of the metal.

We had occasion in the experimental section of this paper to point out that the GSSG used by Hopkins & Morgan [1938] and by ourselves in most experiments, owing to the method of its preparation contained a trace of Cu. The relative slight effect of this on the rate with which such preparations inactivate the succinic enzyme is discussed in the earlier section. That inactivation proceeds typically under the influence of GSSG in the complete absence of Cu is shown in Exp. 8.

It should be noted that inhibition by Cu is reversible and is removed when the enzyme preparation is subsequently incubated with GSH (e.g. Exp. 5).

We have confirmed the results of Morgan & Friedmann who having found that maleic acid and SH-compounds interact to form stable addition compounds, showed that the acid also reacts with the SH groups of proteins and thus inhibits the activity of succinic dehydrogenase. This inhibition is not complete, as an equilibrium is earlier reached. We have found however that maleic acid fails to inhibit appreciably the activities of the lactic, glycerophosphate and malic enzymes, and the same is true—at least under the conditions of our experiments—of iodoacetic acid.

It seemed probable that substances which inhibit the succinic dehydrogenase must enter into such relations with the active structures of the enzyme as would make them capable of protecting it from oxidation. Malonic acid proved to be very effective in such protection. A tissue preparation exposed to GSSG and malonic acid is found, after being washed free from the acid, to have lost none of its original activity. The protection is exerted with very low concentrations (Exp. 10), much lower than those necessary for the inhibition of the succinic dehydrogenase.

Other efficient protectors are the dicarboxylic acids, succinic acid itself, for instance, and fumaric acid. This is in keeping with what is known as to the nature of the compounds which most readily inhibit the enzyme; two acidic groups being usually necessary. Pyrophosphoric acid also protects with somewhat less efficiency. It is of interest to know that Rapkine (personal communication), studying the inhibition of yeast fermentation by iodoacetic acid, has found that the above-mentioned dicarboxylic acids (though also lactic and glycollic acids) prevent this inhibition. He has come to the conclusion that this is because they may protect thiol groups which at some stage are essential for the fermentation process.

As bearing further on the proof that SH groups are essential to the activity of the succinic enzyme we endeavoured to obtain direct evidence that these groups remain intact when preparations are protected on the above lines. This again is made difficult by the circumstance that these groups, as present in the indifferent tissue proteins, are so greatly in excess of those which can belong to the enzyme itself. Some evidence for such protection which seems satisfactory has however been obtained and is described above.

With the evidence now available the significance of thiol groups in the activity of the succinic dehydrogenase scarcely admits of a doubt. In this respect the enzyme stands alone among the typical dehydrogenases so far studied, and it seems probable that its case is unique. It is remarkable that it thus shares a character with certain hydrolases while differing from enzymes with functions analogous with its own. This will add interest to future endeavours to discover the mechanism of its action.

Summary

While the succinic dehydrogenase is inactivated by incubation with GSSG and its activity restored by subsequent incubation with GSH this treatment has no effect on the other dehydrogenases studied.

Alloxan in low concentrations, as an oxidant of SH-groups, inactivates the succinic enzyme but not the other dehydrogenases.

The succinic enzyme is inactivated by Cu and by maleic and iodoacetic acids; substances which react with SH groups. They have little or no effect on the other enzymes tested.

Malonic, succinic and fumaric acids protect the succinic dehydrogenase from the influence of GSSG.

The experiments described support the view that the succinic dehydrogenase requires for its activity the integrity of SH groups in its structure while this is not the case with other typical dehydrogenases.

When muscle tissue is exhaustively extracted with salt solutions dehydrogenases remain active in the residual structure of the fibres, which still shows cross striation and birefringence.

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Note added on 10 October, 1938. After the paper had been read in proof our attention was called to a publication by v. Euler and Hellström (*Ark. Kemi Min. Geol.* 1938, **13** B, 1) in which it is shown that oxidation by ferricyanide reduces the activity of the succinic dehydrogenase which reduction by hydrosulphite increases. This paper has now appeared in the current number of *Hoppe-Seyl. Z.* (**255**, 159).

CCXXXIX. STUDIES IN DETOXICATION

II. (*a*) THE CONJUGATION OF ISOMERIC 3-MENTHANOLS WITH GLUCURONIC ACID AND THE ASYMMETRIC CONJUGATION OF *dl*-MENTHOL AND *dl-iso*MENTHOL IN THE RABBIT. (*b*) *d-iso*MENTHYLGLUCURONIDE, A NEW CONJUGATED GLUCURONIC ACID

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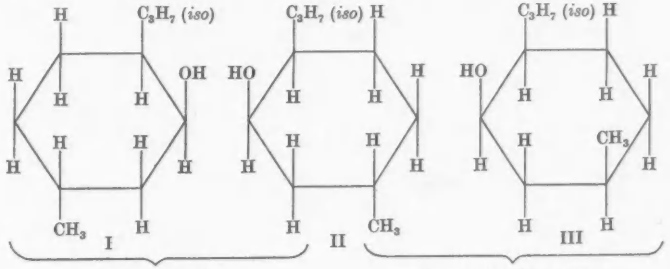
When terpenes (or their derivatives) are administered to animals, a considerable proportion is excreted as hydroxy derivatives conjugated with glucuronic acid; for example, 80 % of borneol is conjugated in this way in man [Pryde & Williams, 1936]. It was from the conjugated glucuronides formed after the feeding of camphor to dogs that *d*-glucurone was first isolated [Schmiedeberg & Meyer, 1879; cf. Jaffe, 1878]. Conjugation with glucuronic acid does not, however, account for all the terpene administered and it is probable that some portion undergoes complete oxidation. This work deals only with the conjugation.

The main objects of the present investigation were to find out whether the optical (enantiomorphic) and geometrical isomerism of the menthols (3-menthanols) influenced their conjugation with glucuronic acid in the body and whether the feeding of a *dl*-menthol resulted in the excretion of a conjugated glucuronide containing more of one antipode than the other.

The conjugation of menthol and iso*menthol*

It has already been shown by Quick [1932] and by Williams [1938] that conjugation can be influenced by position isomerism, i.e. *o*-, *m*- and *p*-substitution. The terpene alcohols, the menthanols, exhibit three types of isomerism, namely structural (e.g. 2-menthanol and 3-menthanol), geometrical (e.g. menthol and *iso*menthol) and optical isomerism (e.g. *d*- and *l*-menthol). The present investigation deals with the 3-menthanols alone. Four[1] of these are theoretically possible, each occurring in *d*-, *l*- and *dl*-forms [Simonsen, 1931]. Only two have been available and these are menthol (I and II) and *iso*menthol (III). The forms used were *l*- and *dl*-menthol and *d*- and *dl-iso*menthol.



[1] Menthol, *neo*menthol, *iso*menthol and *neoiso*menthol.

The mean percentages of these substances conjugated with glucuronic acid in the rabbit under standard conditions (dose 1 g./kg.) are summarized in Table I. The calculated values for *d*-menthol and *l-iso*menthol are also shown.

Table I. *The percentage conjugation of menthol and* iso*menthol with glucuronic acid*

Menthol	% conjugated	*iso*Menthol	% conjugated
l-	48	*l-*	45 (calc.)
dl-	59	*dl-*	55
d-	70 (calc.)	*d-*	65

Table I shows that there is only a slight difference between the conjugation values of menthol and *iso*menthol provided that similar forms are compared, but there is a pronounced difference between those of their optical antipodes. The conjugation of either of the *d*-antipodes is nearly 50 % greater than that of either of the *l*-antipodes. The conclusion is therefore drawn that the conjugation of these two menthanols with glucuronic acid in the rabbit is little influenced by their geometrical isomerism but considerably affected by their optical isomerism.[1] Further, the menthanols possessing the *d*-configuration are more easily conjugated and therefore presumably more resistant to biological oxidation than those of the *l*-configuration.

The asymmetric conjugation of dl-*menthol and* dl-iso*menthol*

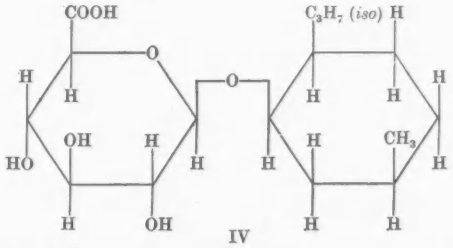
Since one optical isomeride is conjugated to a greater extent than its enantiomorph, it follows that on feeding an optically inactive *dl*-mixture, the excreted glucuronide should contain more of one isomeride than of the other. It has already been shown qualitatively that after feeding *dl*-borneol, *dl-iso*borneol [Hämäläinen, 1909], *dl*-menthol [Jacobsohn & da Cruz, 1929; 1930] or *dl-p-sec*-butylphenol [Fromageot, 1929], these compounds are excreted in conjugated forms which contain unequal amounts of the original *d*- and *l*-compounds. Administration of *dl*-camphor also results in the excretion of an optically active campherol [Meyer, 1908], but in this case the result may be complicated by the fact that at least two camplierols (3- and 5-hydroxycamphors) are formed after the feeding of camphor to dogs [Asahina & Ishidate, 1928]. It has also been shown [Pryde & Williams, 1934] that when commercial *d*-borneol is fed to dogs, the borneol excreted in conjugation with glucuronic acid contains a greater proportion of the *d*-isomeride than that fed. None of this work was quantitative, since no estimations were made of the extent of conjugation of either of the optical antipodes or of the *dl*-compound itself. Further, conjugated glucuronides such as *d*-menthyl-β-*d*-glucuronide and *l*-menthyl-β-*d*-glucuronide, are not optical antipodes and therefore fractional crystallization is possible during the isolation of the mixed glucuronides after the feeding of a *dl*-compound. Hence the amount of each isomeride in the compound isolated may depend to some extent on a difference in solubility between the two glucuronides. Table I shows that *d*-menthol is conjugated to greater extent than *l*-menthol; if *dl*-menthol behaves in the body as a simple mixture of equal proportions of *d*- and *l*-menthol, then the excreted glucuronide after feeding *dl*-menthol should contain more

[1] The term "optical isomerism" is here used in the limited sense of "enantiomorphism". All the geometrical isomerides of 3-menthanol possess, in addition to a cyclic structure, three asymmetric carbon atoms and are optically active. They are therefore, broadly speaking, optical isomerides, but they are are not all enantiomorphs.

d-menthol than *l*-. From the figures in Table I it can be calculated that the menthol recovered after the administration of *dl*-menthol should have $[\alpha]_D$ about $+10°$ in alcohol (taking menthol as $[\alpha]_D \pm 50°$ in alcohol), i.e. about 60 % *d*- and 40 % *l*-. In duplicate experiments values of $+14{\cdot}4°$ and $+12{\cdot}8°$ (in alcohol) were obtained and these figures correspond to *d*- 64 %, *l*- 36 % and *d*- 63 %, *l*- 37 % respectively. A similar argument can be applied to *dl-iso*menthol, the calculated value of the rotation being about $+5°$ (in alcohol) which corresponds to 59 % *d*- and 41 % *l*- (*iso*menthol has $[\alpha]_D \pm 26°$ in alcohol). The figures found experimentally with *iso*menthol were $+6{\cdot}3°$ (i.e. 62 % *d*- and 38 % *l*-) and $+5{\cdot}8°$ (i.e. 61 % *d*- and 39 % *l*-). The optical measurements are therefore in fair agreement with values calculated from the chemical analyses quoted in Table I (see also Table III).

d-iso*Menthyl-β*-d-*glucuronide*

During the progress of this work a new conjugated glucuronic acid has been isolated from the urine of rabbits following the administration of *d-iso*menthol. The probable structure of this product (*d-iso*menthylglucuronide) is shown in the accompanying formula (IV). A description of the acid and some of its derivatives is given in the experimental section.



IV

It has also been found that *dl*-menthol and *dl-iso*menthol give good yields of their respective glucuronides when fed to rabbits. This suggests their possible use as profitable biosynthetic sources of glucuronic acid [cf. borneol, Quick, 1927].

Experimental

Female rabbits, fed on a diet of 100 g. cabbage and 50 g. bran a day, were used under conditions similar to those already described [Williams, 1938]. *dl-iso*Menthol,[1] *l*- and *dl*-menthols were fed in warm water emulsions and *d-iso*menthol in aqueous suspension by stomach tube. The urine was collected for 2 days and analysed for conjugated glucuronic acid by ether extraction in a small continuous extractor (extracting 10 ml. urine in 3 hr.). The ethereal extract was evaporated and the residue hydrolysed by acid and then neutralized. The free glucuronic acid was estimated with Benedict reagent by the Quick method [1925]. The Benedict reagent was standardized against analytically pure *d-iso*menthylglucuronide, $C_{16}H_{28}O_7$, H_2O, and glucose. The ratio of the amounts of glucuronic acid and glucose required to reduce a given volume of Benedict reagent was 1·098 (calc. 1·077). The experimental results for the percentage conjugations of the 3-menthanols are given in Table II, whilst the average figures are given in Table I.

[1] Rotations: $[\alpha]_D$ in alcohol: *l*-menthol (B.D.H.), $-50{\cdot}6°$ ($c = 1{\cdot}4$); *d-iso*menthol (Howard, Ilford), $+25{\cdot}2°$ ($c = 1{\cdot}0$); *dl*-menthol (Howard, Ilford), 0°; *dl-iso*menthol (Howard, Ilford), 0°.

Table II. *The percentage conjugations of menthol and* iso*menthol with glucuronic acid*

Substance	Rabbit no.	Wt. of rabbit (kg.) and dose (g.)	Urine vol. ml.	Glucuronic acid g.	Menthol conjugated g.	Menthol conjugated %
l-Menthol*	2	2	224	0·996	0·801	40
	5	2·4	357	1·393	1·120	47
	6	2·4	358	1·582	1·273	53
	2	2	265	1·345	1·081	54
dl-Menthol	1	2	248	1·514	1·217	61
	2	2	199	1·458	1·173	59
	3	2	254	1·444	1·161	58
*d-iso*Menthol	1	2	224	1·743	1·402	70
	2	2	273	1·567	1·260	63
	1	2	307	1·511	1·214	61
*dl-iso*Menthol	2	2	314	1·484	1·194	60
	1	2	216	1·364	1·097	55
	1	2	190	1·226	0·986	49

* In two experiments with *l*-menthol Quick [1924] found values of 42 and 47·5%, the dose being 2 g.

Experiments on asymmetric conjugation

(*a*) dl-*Menthol*. A rabbit was given 3 g. of *dl*-menthol in warm water by stomach tube and the urine collected for one or two days (see Table III). The urine was made slightly alkaline with NH_4OH and the precipitate of phosphates removed. To the filtrate solid $(NH_4)_2SO_4$ (50 g./100 ml.) was added and the whole boiled and filtered. The filtrate was kept in the refrigerator overnight. The crystalline precipitate of ammonium menthylglucuronate was separated, dried *in vacuo* and weighed. This salt was then hydrolysed by boiling under reflux for 1 hr. with 10% H_2SO_4. The hydrolysed solution was steam-distilled and the distillate cooled in ice. The solidified menthol was collected from the distillate, carefully dried with filter paper and weighed. The M.P. of the recovered menthol was 25–28°. Its specific rotation was measured in absolute alcohol (see Table III).

Table III. *The recovery of menthol and* iso*menthol from urine after the feeding of their* dl-*forms*

Rabbit no.	Substance fed	Dose g.	Urine vol. ml.	Menthol recoverable g.	Menthol recovered in NH_4 salt g.	Menthol from steam distillation g.	$[\alpha]_D$	$[\alpha]_D$ calc.
6	*dl*-Menthol	3	202*	1·77 (59%)	1·6	1·5	+14·4°	+10°
3		3	300†	1·77 (59%)	1·5	1·3	+12·8°	
4	*dl-iso*Menthol	2·5	222*	1·4 (55%)	1·2	1·0	+6·3°	+ 5°
5		3	197*	1·7 (55%)	1·7	1·6	+5·8°	

* In 1 day. † In 2 days. Rotations in absolute alcohol.

(*b*) dl-iso*Menthol*. The procedure used was the same as for *dl*-menthol except that the filtration after adding $(NH_4)_2SO_4$ was omitted. This omission was necessary since ammonium *iso*menthylglucuronate would not completely redissolve in the boiling urine after the addition of $(NH_4)_2SO_4$. The recovered *iso*menthol had M.P. 40–42° (see Table III).

d-iso*Menthyl-β*-d-*glucuronide and its derivatives*

*d-iso*Menthylglucuronide can be isolated from urine directly as the free acid or, after precipitation with $(NH_4)_2SO_4$, as the ammonium salt.

(*a*) *Isolation as free acid.* The isolation of the free acid is an exceedingly simple procedure, since the compound is precipitated from the urine when the latter is acidified. Rabbits were given 2–3 g. of *d-iso*menthol with water by stomach tube. The excretion of the glucuronide was practically complete within 24 hr. The urine was collected after one or two days and, after filtering through cotton wool, was made strongly acid with 20% (wt./wt.) H_2SO_4 (25 ml. acid to 100 ml. urine). Warming to 40° accelerates the separation. The glucuronide began to separate immediately and the process was complete after several hours in the refrigerator. The compound was then filtered under suction, washed with water and dried. In most cases the crude glucuronide had a pinkish colour due to urinary pigments, but in some cases an almost pure white product was obtained. One recrystallization from hot water (charcoal) gave a white crystalline product, M.P. *ca.* 120°. The yield was 0·7 g. per g. of *d-iso*menthol fed (see Table IV).

(*b*) *Isolation as ammonium salt.* The urine was made slightly alkaline with ammonia, boiled and the precipitate of phosphates removed by filtration. To the filtrate solid $(NH_4)_2SO_4$ was added (30 g./100 ml.) and the solution boiled. Immediate precipitation of the ammonium *d-iso*menthylglucuronate took place and the process was completed in the refrigerator. The crude salt was then separated and dried. The yield was 1·2 g. per g. of *d-iso*menthol fed. The recovery of material was better than by the first method (see Table IV).

Table IV. *The recovery of* d-iso*menthylglucuronide from urine*

Rabbit no.	Dose g.	Yield as free acid g.	Rabbit no.	Dose g.	Yield of NH_4 salt g.
1	2	1·6	5	2·5	3·0
1	2	1·4	6	2·5	2·8
2	2	1·0	7	2·5	3·2
4	2·5	1·75			
6	2·5	1·8			

d-iso*Menthyl-β*-d-*glucuronide.* The crude glucuronide after two recrystallizations from a large volume of hot water was obtained pure. It forms long fine matted needles, M.P. 126° (corr.); it is very sparingly soluble in cold water, but easily soluble in hot water, ethyl alcohol, methanol and ether. It crystallizes from water with $1H_2O$ and has $[\alpha]_D^{16°} -43·2°$ ($c = 1·2$ in abs. alcohol). (Titration: 114·75 mg. required 16·48 ml. of 0·0198 *N* NaOH; calc. 16·55 ml. Found: C, 54·75; H, 8·6; H_2O, 5·0%. $C_{16}H_{28}O_7$, H_2O requires C, 54·8; H, 8·6; H_2O, 5·1%.)

Ammonium d-iso*menthyl-β*-d-*glucuronate.* The crude ammonium salt was purified by dissolving in hot water, filtering and precipitating with $(NH_4)_2SO_4$. Three such treatments gave an analytically pure salt as glistening plates. The salt was anhydrous and had $[\alpha]_D^{18·5} -41·1°$ ($c = 1·3$ in water). (Found: N, 4·02%. $C_{16}H_{31}O_7N$ requires N, 4·01%.)

Cadmium d-iso*menthyl-β*-d-*glucuronate.* Aqueous solutions of the ammonium salt gave amorphous precipitates with soluble Cu, Fe, Zn, Ag and Co salts. On adding aqueous $CdCl_2$ to a solution of the above ammonium salt and slightly warming the mixed solutions, an insoluble crystalline cadmium salt separated as shining platelets. (Found: Cd, 14·7%. $(C_{16}H_{27}O_7)_2$ Cd requires Cd, 14·5%.)

Hydrated cadmium salts of *l*-menthylglucuronide have been prepared by Fromm & Clemens [1901], but the present compound was anhydrous and lost no weight at 110°.

The recovery of conjugated glucuronic acid after the feeding of dl-*menthol and* dl-iso*menthol*

By using the $(NH_4)_2SO_4$ precipitation method already described, good yields of crude ammonium menthyl- and *iso*menthyl-glucuronates can be obtained from the urines after feeding the *dl*-compounds. The yields were 1·1 to 1·4 g. per g. of the *dl*-menthanols fed (see Table V).

Table V. *Yields of glucuronides after* dl-*menthol and* dl-iso*menthol*

Exp.	Substance	Dose g.	Yield of crude NH_4 salt g.
1	*dl*-Menthol	3	4·1
2	,,	3	3·3
3	,,	2	2·5
4	*dl-iso*Menthol	2·5	3·0
5	,,	3	4·4

These ammonium salts are of course mixtures containing more of the glucuronides of the *d*- than of the *l*-isomerides.

The glucuronide after feeding dl-*menthol.* Some of the crude ammonium salt obtained after feeding *dl*-menthol was further purified and converted into the free acid by adding the requisite amount of dilute HCl. The free acid was recrystallized from hot water, crystallization being accelerated by adding a little dilute HCl to the cooled solution. One purified specimen had M.P. 103–104° and $[\alpha]_D^{19°}$ −41·8° (*c*=2 in alcohol) (pure *l*-menthylglucuronide has $[\alpha]_D$ −105° in alcohol [Fischer, 1910]). Like the pure *l*-derivative this mixture crystallizes with 1·5 H_2O. (Titration: 108·6 mg. required 15·16 ml. of 0·01994 *N* NaOH; calc. for $C_{16}H_{28}O_7$, 1·5 H_2O, 15·17 ml.)

The glucuronide after dl-iso*menthol.* A specimen of the crude ammonium salt recovered after the feeding of *dl-iso*menthol was subjected to two further recrystallizations from hot half-saturated $(NH_4)_2SO_4$ solution. The purified material had $[\alpha]_D^{19°}$ −54·4° (*c*=1·6 in water); pure ammonium *d-iso*menthyl-glucuronate has $[\alpha]_D$ −41° (in water).

It must be pointed out, however, that *d*- and *l*-menthyl- (or *-iso*menthyl-) glucuronides are not optical antipodes and therefore the purification of the mixed free acids or ammonium salts will result in some measure of fractional crystallization. The measured specific rotations will therefore be somewhat variable. Experience gained in this work shows that the *l*-compounds are more soluble than the *d*-menthylglucuronides. The separation of the *l*- and *d*-menthyl-glucuronides is being further investigated.

Summary

1. The extents of conjugation of the geometrical isomerides, menthol and *iso*menthol, with glucuronic acid in the rabbit are almost identical, provided that similar optical isomerides are compared. The optical isomerides (enantiomorphs), *d*- and *l*-menthol and *d*- and *l-iso*menthol show pronounced differences in conjugation, the *d*-compounds conjugating to nearly a 50% greater extent than the *l*-.

2. Asymmetric conjugation with glucuronic acid takes place when *dl*-menthol and *dl-iso*menthol are fed to rabbits. The menthol and *iso*menthol recovered from the urine have positive rotations which agree with the values calculated from the percentage conjugations of the separate *d*- and *l*-isomerides.

3. The preparation and properties of *d-iso*menthyl-β-*d*-glucuronide and of two of its derivatives are described for the first time.

4. It is suggested that *dl*-menthol and *dl-iso*menthol can be used as profitable sources of biosynthetic glucuronic acid.

The *dl*-menthol, *d*- and *dl-iso*menthol used in this work were supplied by Messrs Howard and Sons, Ltd., Ilford.

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CCXL. STUDIES IN THE SECRETION OF MILK FAT

I. THE EFFECT OF INANITION ON THE BLOOD LIPOIDS OF THE LACTATING COW

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During the past few years the work of Lintzel [1934], Blackwood [1934], Graham *et al.* [1936] and Maynard *et al.* [1938], based on the principle of simultaneous arterial and venous blood sampling, has shown that the only lipoid fraction of the blood used by the mammary gland for milk fat production is the neutral glyceride fraction and not that comprising the phosphatides or cholesteryl esters. At first sight this might seem to suggest that for the secretion of milk fat the gland simply selects from the blood the particular type of glycerides which it requires and that these are then allowed to diffuse into the milk. But it is clear from the peculiar composition of the fat itself and also from the fact that the respiratory quotient of the actively lactating mammary gland has now been found by Graham *et al.* [1938] to be considerably greater than unity, that more complex processes such as the synthesis of fat from carbohydrates are also undoubtedly involved. It would therefore appear that there are at least two main precursors of milk fat in the blood, the neutral glycerides on the one hand and some form of carbohydrate material on the other, and so it seems reasonable to suppose that the composition of the milk fat produced by an individual animal at any particular time will depend on the extent to which each of these precursors contributes towards its production. It was felt that further evidence regarding the biochemical and physiological processes involved in milk fat secretion might be obtained by a detailed study of both the blood and milk fats of animals kept under such experimental conditions as would be expected to alter the proportions in which these two precursors were used by the gland. One possible method of achieving this end would be to cause in some way a marked alteration in the lipoid concentration of the blood of the cow in the hope that an increase or decrease in the level of triglycerides in the plasma would considerably alter the proportion of the milk fat arising from this particular fraction of the blood. Consequently, it was decided to investigate the problem by experiments involving inanition and certain other procedures which might be expected to cause a considerable alteration in the relative availabilities of the two precursors. The present paper and the communication which follows it are concerned only with the inanition work, the first dealing exclusively with the blood lipoids and the second with the milk fat. The effect of inanition on the general composition of the milk has already been discussed by Smith *et al.* [1938].

Experimental

The blood and milk fat samples analysed in the present work were obtained in the course of an experiment undertaken by Dr S. Morris to determine the effect of inanition on the metabolism of lactating and non-lactating ruminants. The animals used were three non-pregnant Ayrshire cows in the sixth to seventh month of their lactation period. After a few weeks, during which they had been receiving a normal well-balanced diet, food was withheld from two of them for a period of 12 days, after which free access to food was again permitted, water being given freely at all times. Under these circumstances Cow no. 1 appeared to present a case of simple inanition throughout the 12 days, but with Cow no. 2 severe symptoms of milk fever occurred in the second day of the fast, and although they rapidly disappeared after inflation of the udder, their occurrence must be taken into consideration as a possible complicating factor. For Cow no. 3 the experiment was discontinued on the seventh day of the fast because by that time the condition of the animal was not satisfactory, a fact which may well have been due to a marked deficiency in depot fat as revealed by subsequent *post mortem* examination. At suitable times before, during and after inanition, blood samples were taken from one of the mammary veins, and composite daily milk samples were collected for the isolation of the fat.

Extraction of the blood lipoids

About 200 ml. of blood were centrifuged for 45 min. and the plasma was separated from the corpuscles. The plasma was then allowed to drop in a fine stream into about 15 volumes of alcohol-ether (3 : 1), the amount of solvent made up to 20 volumes and the mixture left for a few hours at room temperature with occasional shaking. It was then filtered, aliquots of the filtrate were removed for the colorimetric estimation of phosphorus and a known volume of the filtrate, usually about 2 l., was reduced to dryness on a water bath *in vacuo* under an atmosphere of nitrogen. The residue so obtained was extracted several times with chloroform and the extracts were filtered from the relatively large amounts of blood inorganic salts which accumulated at this stage. The corpuscles were similarly treated except that they were first mixed with an equal volume of water to cause haemolysis and the mixture was finally made up in 25 volumes of alcohol-ether. 20 volumes of solvent were used for the plasma and 25 for the corpuscles, since it has recently been shown by Boyd [1936, 1, 2] that these are the minimum proportions which can be used with safety if the fat extraction in both cases is to be as complete as possible.

Analysis of the blood lipoids

Owing to the fact that the volume of blood available in most animal experiments and also the concentration of lipoids in the blood are both so exceedingly small, accurate analysis of the blood lipoids has always been a matter of considerable difficulty. In the present work, for reasons outlined later, it was decided not to employ any of the usual micro methods, but rather to take blood samples of such a size (about 200 ml.) that the ordinary standard macro procedures, suitably adapted for use with unusually small amounts, could be applied to the resulting lipoids. This was possible in the present work since the withdrawal of such large samples of blood is without ill effect on the cow. For every sample, therefore, fatty acids and total unsaponifiable matter were determined by direct weight, cholesterol by weighing the digitonide, iodine values by the

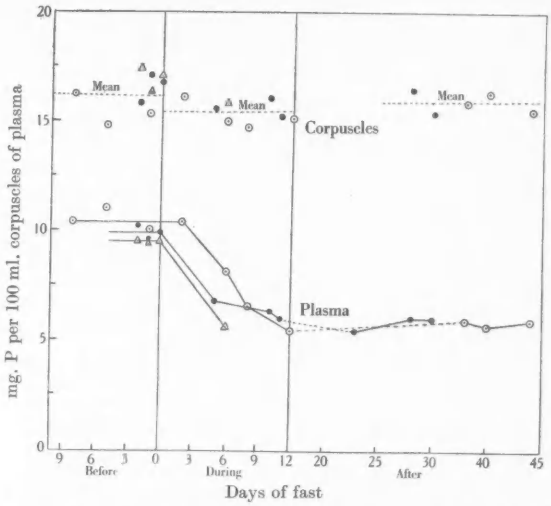

Fig. 1. The effect of inanition on the lipoid phosphorus content of the blood. •—• Cow no. 1. ⊙—⊙ Cow no. 2. △—△ Cow no. 3.

Table I. *The analytical data for the corpuscle lipoids (mg./100 ml.)*

Days of fast		Total fatty acids			Total unsaponifiable matter	Cholesterol	Cholesterol content of unsaponifiable matter
		Wt.	I.V.	Mol. wt.			
				Cow no. 1			
Before	2	—	63	354	—	—	—
	1	374	60	360	227	183	81
	0	400	63	379	222	186	84
During	5	381	60	367	225	186	82
	10	406	59	349	222	192	86
	11	359	60	363	216	186	86
After	28	355	63	359	217	174	80
	30	363	62	342	188	165	88
				Cow no. 2			
Before	8	388	67	380	228	190	83
	5	404	65	363	207	180	87
	1	400	67	377	215	183	85
During	2	397	66	391	236	200*	85
	6	372	61	341	214	178	83
	8	386	56	—	220	176	80
	12	371	59	350	205	188	91
After	38	362	59	367	201	167	83
	40	378	62	340	213	171	80
	44	371	60	359	222	172	78
				Cow no. 3			
Before	2	432	64	336	207	196	95
	1	413	63	398	216	187	88
	0	408	64	334	206	193	94
After	6	389	59	364	214	196	92

* Day on which Cow no. 2 suffered from milk fever.

bromine absorption method and molecular weights by titration. Many precautions are necessary if accurate results are to be obtained, but as it is hoped that a separate communication on the details of the procedure will shortly be published, only the barest outline of the methods need be recorded here.

Corpuscles. As the corpuscle lipoids consist almost entirely of free cholesterol and phosphatides it was felt that nothing would be gained by the preliminary separation of the phosphatides. The solvent was therefore removed from the chloroform solution, obtained as already described, and the residue saponified directly by heating it for about 30 min. with 20 ml. alcohol and 2 ml. 40 % KOH, to which 20 ml. benzene were added to render the phosphatides more soluble. The fatty acids and unsaponifiable matter were then isolated, washed with water and finally made up to known volumes in light petroleum, from which suitable aliquots were taken for analysis. The results for lipoid P as estimated in the original alcohol-ether extract are shown in Fig. 1, while the remaining values are recorded in Table I.

Plasma. After the removal of the solvent from the chloroform solution of the plasma lipoids, the residue was dissolved in about 10 ml. of ether and transferred to a centrifuge tube and the phosphatides were precipitated with 30 ml. acetone. After standing at room temperature, the mixture was centrifuged, the clear supernatant liquid poured off and the residue washed with acetone. It was then dissolved in ether and reprecipitated. Finally, both the phosphatide and non-phosphatide fractions were made up to known volumes and analysed. That almost a complete separation of the two fractions had been obtained was indicated by the facts that the P content of the non-phosphatide fraction was not more than 3 % of the whole and was often considerably less and that no more than the merest traces of cholesterol remained in the phosphatides. For this precipitation the use of $MgCl_2$ and a temperature of 0° are frequently recommended in the literature in order to assist the separation of the phosphatides, but it was found in the present work that these extra details were unnecessary, provided that really pure ether and acetone were available, and, as there is a very serious risk of precipitating some of the cholesteryl esters and neutral glycerides along with the phosphatides at such a low temperature as 0°, and as Mg ions may tend to cause difficult emulsions at a later stage, the simpler procedure outlined above was found to be the safest and most efficient.

After the P estimation, the phosphatide fraction was saponified as already described for the corpuscles. Total fatty acids and unsaponifiable matter were then isolated and analysed. For the non-phosphatide fraction, total lipoids and free cholesterol were first estimated in suitable aliquots before the remainder was saponified. The variations in concentration of the different plasma lipoids for the three animals are shown in Figs. 1, 2 and 3, while the molecular weights and iodine values of the acids are arranged in Table II.

One of the chief difficulties in analysing blood lipoids at the present time is that no method has yet been found for separating the cholesteryl esters from the neutral glycerides, these fractions, which together form the two main constituents of the non-phosphatide fraction, having very similar solubility properties. It is only possible to estimate the amount of fatty acids originally present as triglycerides by calculation. This was done here by assuming that the mean molecular weight actually found for the total non-phosphatide fatty acids was also the mean molecular weight of the acids esterified with cholesterol, which, as the latter greatly predominate, would give a very close approximation to the truth. From the amount of glyceride fatty acids so estimated it was possible to calculate the amount of glycerol which must have been required for

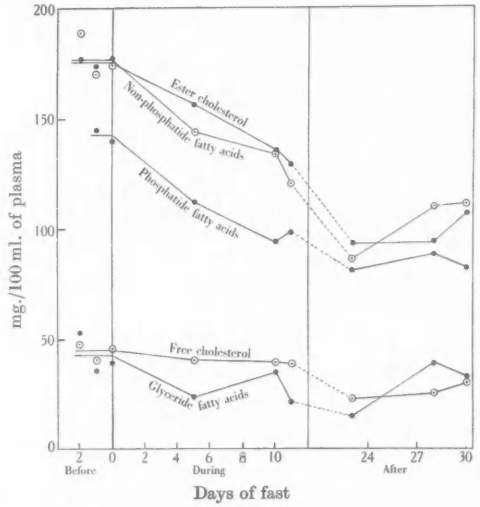

Fig. 2. The effect of inanition on the plasma lipoids of Cow no. 1.

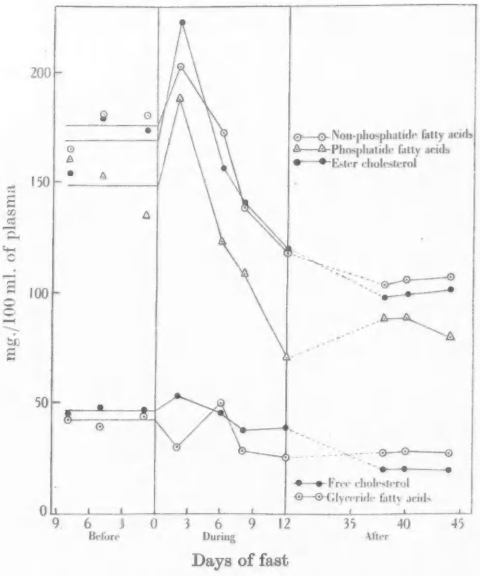

Fig. 3. The effect of inanition on the plasma lipoids of Cow no. 2.

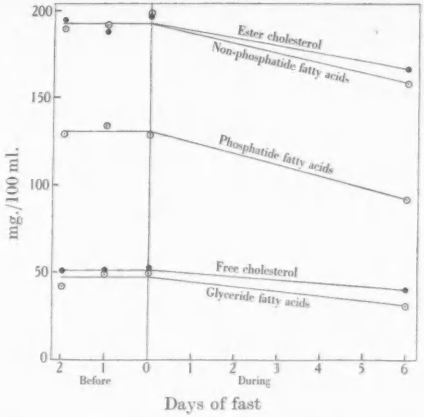

Fig. 4. The effect of 6 days' inanition on the plasma lipoids of Cow no. 3.

Table II. *Analytical data for the plasma lipoids not shown diagrammatically in the Figs.*

Days of fast		Fatty acids: Phosphatide I.V.	Fatty acids: Phosphatide Mol. wt.	Fatty acids: Non-phosphatide I.V.	Fatty acids: Non-phosphatide Mol. wt.	Total cholesterol content of unsaponifiable matter %	Sum of fatty acids, unsaponifiable matter and glycerol as % of original non-phosphatide fraction
				Cow no. 1			
Before	2	64	333	134	295	99	97
	1	66	359	136	295	94	95
	0	59	335	135	293	94	95
During	5	62	320	136	295	91	96
	10	65	340	125	304	96	94
	11	62	359	134	291	96	90
After	23	62	322	137	294	85	94
	28	74	323	129	294	91	94
	30	74	340	136	295	92	94
				Cow no. 2			
Before	8	60	342	139	304	90	99
	5	71	331	142	305	96	99
	1	60	318	137	312	97	99
During	2	68	332	149*	306	97	97
	6	69	388	137	303	94	97
	8	70	333	140	304	91	99
	12	58	370	137	297	93	94
After	38	63	355	130	301	88	92
	40	57	347	131	302	90	94
	44	65	348	130	304	90	88
				Cow no. 3			
Before	2	77	333	140	293	98	98
	1	69	323	143	289	97	96
	0	65	366	142	293	98	97
After	6	59	370	143	294	99	93

* Day on which Cow no. 2 suffered from milk fever.

their esterification. Then, by adding together the values found for total fatty acids, total unsaponifiable matter and those calculated for glycerol, it was possible to estimate the percentage of the original non-phosphatide fraction which was accounted for by the various constituents. It will be observed from Table II that this figure was usually well over 90%, a fact to which reference is made later.

Discussion

The work of Maynard & McCay [1929] and of Porcher & Maynard [1930] has shown that the blood lipoid level of ruminants under normal circumstances changes extremely slowly and that variations in it tend to be but slight. In fact with non-lactating cows Aylward & Blackwood [1936] found no definite or consistent alteration in the total fatty acid content of the blood even after a fast of $10\frac{1}{2}$ days, while later results of Aylward *et al.* [1937] showed that although for two days after a drench of "labelled" fat the blood of the cow became gradually enriched with the "labelled" constituents of the drench, the actual lipoid level itself was not measurably affected. It was therefore obvious that if the small changes in composition or in amount which may be expected in the blood fat of ruminants were to be detected, the methods adopted for analysis would have to be as searching and as accurate as possible. For this reason it was felt that the micro procedures now commonly in use such as that of Bloor [1928; 1929], were not applicable to the present experiments as they would not deal with sufficient material for the thorough examination which the author had in view. Nor was another type of method such as that published by Stoddart & Drury [1929] of much value here, as it depends on the assumption that the mean molecular weight of the fatty acids is always a given value from which their amount is calculated, whereas actually in the present work, the constancy or variability of these particular molecular weights before and during inanition was one of the properties which it was desired to investigate. It was therefore decided that the macro procedure, suitably adapted, would be the one most likely to give the best results. Although this method is somewhat long and laborious, it was believed that its application to a few large blood samples would be of much greater value than more numerous but less reliable analyses carried out on very much smaller amounts.

Phosphorus. The lipoid P results shown in Fig. 1 are typical of the general effect of inanition found for the other lipoid fractions of the blood. In the corpuscles the average figure of 16·2 mg. per 100 ml. for all three cows prior to the fast fell by less than 5% to 15·4 during inanition, and a few weeks after re-alimentation it had tended to return towards the original value. That it never quite reached the initial figure may well have been due in part to the presence of small amounts of plasma still remaining in the corpuscles, for the latter were not washed with saline prior to their extraction as it was felt that, by prolonging the process of separation unnecessarily, more errors might be introduced than were actually avoided and that any plasma still remaining in the corpuscles would be too small to invalidate the general conclusions in any way.

In the plasma very much greater changes were observed than in the cells. The mean initial figures of about 10 mg. were reduced by almost 40% to 6 mg. during inanition, and strangely enough this low level persisted even 6 weeks after free access to food was again permitted. In the work of Aylward & Blackwood [1936], the lipoid P level in whole blood was found in most cases to rise immediately after food was withheld rather than to fall, but this unexpected observation can now be explained by the facts that almost half the lipoid P in whole blood is

contained in the corpuscles, that its level in the corpuscles is not much affected by inanition and that the proportion of corpuscles in the blood as shown by Smith *et al.* [1938] increases from about 30 to over 40 % in the initial stages of starvation. It is therefore only later in the fast that an appreciable reduction in the phosphatide content of whole blood is noticeable. It is of interest to observe that the total decrease in the lipoid P of whole blood found for dry cows by Aylward & Blackwood [1936] was about 10 % as compared with the much larger figure of 25 % which can be calculated for lactating cows in the present work, notwithstanding the fact now well established that phosphatides are not precursors of milk fat.

Corpuscles. One of the main objects of analysing the lipoids of the corpuscles was to find whether their level or nature was materially affected by very abnormal conditions, for if no more than minute changes could be observed under such a prolonged period of inanition, it is highly improbable that they would ever change appreciably under any normal circumstances to which the cow might be subject. Consequently, it would be most unlikely that variations in milk fat secretion, whatever their cause, could ever be traced to changes or temporary peculiarities in the lipoids of the cells. If this were in fact found to be so, extension of the corpuscle side of the work in future experiments of this type would be quite unnecessary.

Before discussing the results it is essential to mention that the complete extraction of the lipoids from corpuscles, even by the procedure recommended by Boyd [1936, 2], is by no means easy, nor can it readily be done with identical results for different samples taken at the same time from the same batch of corpuscles. This difficulty can doubtless be attributed to the fact that the cells contain such extremely small amounts of very complex phosphatides in the presence of relatively large quantities of proteins, with which they may even be loosely combined. It is therefore not surprising to find considerable variation in the fatty acid levels before inanition as recorded in Table I, in spite of the fact that there was probably little actual change in the pre-fast period. There is, however, sufficient consistency to show that on the whole there was a reduction of some 6 or 7 % in the total fatty acids as a result of inanition and that this decrease persisted for at least 6 weeks after re-alimentation. Thus with Cow no. 2 an original mean of 397 mg. fell to 371 mg. by the 12th day of the fast and was still close to this value 40 days later. That it did not show any tendency to return towards the original figure in the post-fast period, as was the case with the corpuscle P, was again probably due, in part at least, to the presence of small amounts of plasma still remaining in the corpuscles. As these small residual amounts of plasma had a higher ratio of fatty acids to lipoid P than was the case for the corpuscles themselves, they would have a greater effect on the figures for corpuscle fatty acids than on those for the lipoid P.

With regard to the nature of the corpuscle fatty acids, the iodine values in Table I suggest a very slight reduction in the degree of unsaturation during inanition. Thus, the mean initial figures of 62, 66 and 64 for the three cows fell to 60, 59 and 59 respectively towards the latter part of the fast, and showed a slight tendency to return towards the normal values in the post-fast period, but it is doubtful if such small variations have any significance. Unfortunately, from the molecular weights found for the acids little can be concluded. It appeared during the course of the experiments that the estimation of these particular molecular weights by titration is by no means a simple matter and that this is not entirely due to the very difficult end points which were encountered. The figures obtained ranged from 334 to 391 and possess at least two points of

interest: firstly, the great and irregular variations, and secondly, the magnitude of the values as a whole. From other so far unpublished work by the present author, it may be assumed that the corpuscle phosphatides are much more complex than is generally realized, but even if their main constituents were C_{20}, C_{22} and C_{24} acids, the mean molecular weight of all the acids would not be expected to be much greater than about 340. In fact one experiment on a much larger scale than the present has suggested that the value for corpuscle phosphatide fatty acids from a pooled sample of bovine blood is somewhat under 330. It is hoped that future work on the nature of these phosphatides will throw more light on this aspect of the subject, but at present it can only be stated that the variations in molecular weights as quoted in Table I are probably due to the difficulty of isolating the substances in a pure state and of making the actual determinations, and not to any significant change in the acids themselves.

Turning now to the cholesterol and total unsaponifiable matter, it is again seen from Table I that the alteration in level during inanition was in all cases negligible, but 30 or 40 days after the fast the cholesterol appears to have been somewhat reduced. Thus, taking Cow no. 1 as an example, the pre-fast figure of about 185 remained almost unchanged at a mean of 188 during starvation but then fell by some 9% to 170 in the post-fast period. The reason for the lack of change during the fast and the decrease following re-alimentation cannot at present be given, but taking the results as a whole and comparing them with those of the plasma, these changes in level may be regarded as exceedingly slight.

As the unsaponifiable portion of blood lipoids is frequently assumed to consist almost entirely of cholesterol it was of considerable interest to estimate the actual proportion of each sample which consisted of this compound. It is clear from Table I that for Cows nos. 1 and 2 the mean figure for the amount of cholesterol in the unsaponifiable matter was 85% and for Cow no. 3, 92%, but the individual samples showed too much variation amongst themselves for any conclusion to be drawn as to the effect of inanition upon them. It is clear, however, that in every sample of unsaponifiable matter there was an appreciable amount of material soluble in light petroleum not precipitated by digitonin and not altered by resaponification. From these results as a whole it may therefore be concluded that the corpuscle lipoids of the lactating cow are not readily inclined to alter either in their nature or in their concentration even during prolonged inanition.

Plasma. Unlike the corpuscles, the plasma showed large reductions in the level of the various lipoid constituents as a result of starvation. With Cow no. 1, for example, there was a decrease during the fast of about 26% in ester cholesterol, an initial figure of 176 mg. falling to just below 130 mg., while in the post-fast period this value fell still further to 94 mg., a total decrease of 47%. Similarly with the phosphatide and non-phosphatide fatty acids, original figures of 143 and 177 became 94 and 119 towards the end of the fast and diminished further after re-alimentation to mean values of 83 and 105 respectively. These figures represent a total reduction of 45 and 40% in the phosphatide and non-phosphatide fatty acids between the pre- and post-fast periods. The fact that these values were actually lower some weeks after free access to food had been permitted than they were when food was completely withheld is of considerable interest, though no definite explanation can at present be given. It may be that on re-alimentation, fat was so rapidly being used for replenishing the animal's depleted storage depots and for milk secretion that the level in the plasma could not be maintained above this low figure.

With Cow no. 2 the effects of inanition were complicated on the 2nd and 3rd days by the occurrence of milk fever, which was found to be accompanied by hyperlipaemia. Thus, as shown in Fig. 3, there were increases in the ester cholesterol, phosphatide and non-phosphatide fatty acids of 32, 25 and 14% respectively, initial values of 169, 149 and 178 increasing to 223, 198 and 203. After the symptoms of milk fever had disappeared, however, these increases were followed by considerable decreases analogous to those found for Cow no. 1. Thus for ester cholesterol the value of 223 fell to 117 by the last day of the fast and was reduced to 100 in the post-fast period, a total decrease equal to 41% of the initial pre-fast figure. Similarly the phosphatide and non-phosphatide fatty acids fell to 70 and 118 by the end of the starvation period and were as low as 85 and 106 some weeks after re-alimentation had been established, figures which are equivalent to decreases of 44 and 40% respectively between the initial and final values. Again in Fig. 4 (Cow no. 3) it is confirmed that inanition causes a definite decrease in the various fatty constituents of the plasma, but for this cow the decrease only amounted to about 13% for ester cholesterol, 30% for phosphatide fatty acids and 18% for those from the non-phosphatide fraction by the 6th day of the fast when the experiment was discontinued. It is of interest to notice from the figures just cited that in all cases, whether the lipoid level rose or fell, the phosphatide fatty acids tended to be affected to a somewhat greater extent than the others, perhaps owing to a greater degree of metabolic activity on the part of these particular acids.

Further reference to Figs. 2, 3 and 4 shows that there is also a very definite reduction in free cholesterol and in triglyceride fatty acids as a result of inanition. For Cow no. 1, for example, initial figures of 45 and 43 respectively were reduced to 38 and 21 by the 11th day of the fast and to mean values of 25 and 29 in the post-fast period, final decreases which are equivalent to 44 and 33% of the original pre-fast figures. The corresponding reductions in free cholesterol and glyceride fatty acids for Cow no. 2 were 59 and 36%, and for Cow no. 3, after a fast of only 6 days, they were 22 and 35% respectively. The values for glyceride fatty acids in both Figs. 2 and 3 appear to be characterized by greater irregularities than any of the other lipoid constituents, and although this may actually have been the case in the plasma, it may well have been due mainly to the fact that these values were obtained, as already described, by calculation from the figures found for free and total cholesterol and for total non-phosphatide fatty acids. Any unavoidable errors therefore in the estimation of these latter fractions may have combined in several cases to give somewhat greater errors in the relatively small values calculated for the triglyceride acids. This suggestion gains support from the fact that no such irregularities occurred in the values for free cholesterol, which are almost equally small but which were found by direct estimation.

One point of considerable interest which may be stressed at this stage and which is very obvious from Figs. 1–4 is the extraordinary tendency in the plasma of the cow for all the various lipoid constituents, with the possible exception of the triglycerides, to behave alike under different conditions. Thus in milk fever, the rise in phosphatide fatty acids was accompanied by similar rises in cholesteryl esters and free cholesterol, while again during simple inanition they all decreased in much the same way, with the result that the general composition of the total lipoids tended to remain the same although their level in the plasma changed. This tendency to a constant composition for the lipoids of cow's plasma is exceedingly important, for, if general, it means that in large scale experiments to discover, for example, whether there is any correlation between the characteristic blood

fat level of an animal and its milk fat production, it might be possible in some sections of the work to estimate only lipoid P and cholesterol, both of which can readily be determined, while the more laborious and less accurate estimation of the fatty acids could be omitted, at least in a preliminary survey.

With regard to the effect of inanition on the actual nature of the various lipoid fractions, it may be concluded, just as for the corpuscles, that there was extremely little change. For the phosphatide fatty acids, the iodine values and molecular weights recorded in Table II show too much variation amongst themselves for any definite conclusion to be drawn, except that, considered as a whole, they do not suggest any noticeable alteration as a result of fasting in the general type of fatty acids present. Here again, as with the corpuscles, considerable difficulty was experienced in estimating the molecular weights of these particular fatty acids, so that the variations shown for them are thought to be due mainly to the experimental difficulties involved rather than to changes in the acids themselves. Curiously enough no such difficulties were encountered in estimating the molecular weights of the non-phosphatide fatty acids, for which the values were much more consistent. Thus with Cow no. 1, for example, the molecular weights of the phosphatide acids varied between 320 and 359, whereas for those from the non-phosphatide fraction, they all fell within the limits of 291 and 304, 7 out of the 9 being between 293 and 295. Why there should be this particular difficulty with the acids from the blood phosphatides is not at present clear, but it is hoped that future work will help to elucidate the problem. It is interesting to observe that the non-phosphatide fatty acids of Cow no. 2 had consistently higher molecular weights than those of the other two cows, the mean value being 305 for no. 2 as compared with 295 and 292 for nos. 1 and 3. Some individual variation is obviously possible in this respect.

A study of the data given in Table II for the iodine values of the non-phosphatide acids again suggests that inanition must have caused very little change, if any, in the nature of the acids. It is only with Cow no. 2 that any slight alteration can be seen, for on the day when this animal suffered from milk fever the initial mean iodine value of 139 rose to 149. It then fell again to 138 during the latter part of inanition and ultimately to 130 some 6 weeks after re-alimentation. It would appear therefore that for this animal hyperlipaemia was accompanied by an increase in unsaturation, suggesting the mobilization of unsaturated acids in the emergency caused by milk fever, and that hypolipaemia in the post-fast period was accompanied by a decrease in unsaturation, but at the most these changes were small.

It is a well known fact that it is possible for the iodine values and molecular weights of two mixtures of fatty acids to be the same in spite of differences in the composition of the mixtures. Consequently, where possible, it is advisable, as a third check, to estimate the amounts of saturated acids in the mixtures by the Twitchell separation process. Owing to the small amounts of acids available in the various samples in the present work it was only possible to apply this separation on a micro scale to pooled samples of the plasma non-phosphatide acids from Cow no. 1. The saturated acids so estimated amounted to 11·2% of the total acids before and during inanition and to 12·4% in the post-fast period. These figures may possibly be a little lower than the true values, as they were obtained on very small amounts of material, but they confirm the absence of any appreciable change as a result of the fast.

The proportion of cholesterol in the unsaponifiable matter of the plasma was somewhat higher than for the corpuscles. For example, for Cow no. 2 the figure for the plasma samples varied between 88 and 99% with a mean of 96, whereas

in the corpuscles the limits were 78 and 91 with a mean of 84. Only during the post-fast period of the experiment were any appreciable changes noticed in these particular figures for the plasma. Thus with Cows nos. 1 and 2, pre-fast means of 96 and 94 % both remained almost unaltered during inanition but fell later to 89 %. It can scarcely be said, however, that such small decreases are significant.

In Table II it will be observed that for each sample the non-phosphatide fatty acids and unsaponifiable matter determined by direct weight have been added to the small amount of glycerol calculated to be present (usually about 3 mg./100 ml. plasma), and the sum has been expressed as a percentage of the original non-phosphatide fraction. The great majority of the figures so obtained are found to fall between 94 and 99 % suggesting that the amount of water-soluble fatty acids in the non-phosphatide fraction of the plasma is exceedingly small, if, indeed, they occur there at all. This finding is not in any way at variance with that of Stewart & Hendry [1935] who were able to suggest by other means that the water-soluble fatty acids of whole blood did not exceed 3 % of the total fatty acids.

Summary

1. The effect of inanition on the blood lipoids of three lactating cows has been investigated in detail as part of a scheme for the study of certain problems connected with milk fat secretion.

2. Only very slight decreases of considerably less than 10 % were observed for the lipoid levels in the corpuscles, during and after a 12-day fast, whereas for the plasma, general reductions of as much as 40 or 50 % took place and these low levels persisted even 6 weeks after free access to food had again been permitted.

3. Neither in the corpuscles nor in the plasma could any appreciable change be detected in the actual nature of the lipoids as a result of inanition, although there was a tendency for greater unsaturation in the plasma fatty acids to accompany milk fever symptoms.

4. It was shown that if any water-soluble fatty acids are present in the non-phosphatide fraction of the plasma of lactating cows the actual amount must be exceedingly small.

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CCXLI. STUDIES IN THE SECRETION OF MILK FAT

II. THE EFFECT OF INANITION ON THE YIELD AND COMPOSITION OF MILK FAT

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In the course of research work on the secretion of milk fat, the effect of inanition on the lipoids of both the blood and the milk of actively lactating cows has been studied in detail. The previous paper records the experiments on the blood lipoids, while the present communication describes the changes brought about in the fat of the milk during the fast. At the same time certain modifications are described for the analysis of butter fat by the fractional distillation process.

Experimental

The yield of fat was estimated daily by noting the yield of milk and determining its fat content by the Gerber process. The results are recorded in Fig. 1.

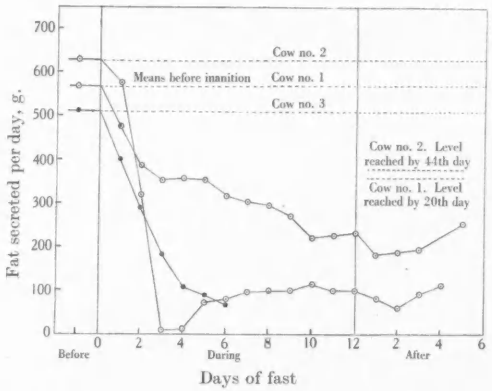

Fig. 1. The effect of inanition on the total daily yields of milk fat. The extremely low yield from Cow no. 2 on the 3rd and 4th days was due mainly to the incidence of milk fever.

For the determination of iodine and Reichert Meissl values, small samples of the milk fat were prepared daily by centrifuging some of the milk, chilling the resulting product in the refrigerator and carefully removing the cake of fat so formed from the top of the aqueous phase. The fat was then further purified by suitable treatment with light petroleum. The larger samples essential for fractional distillation were prepared by separating the milk, churning the

cream and purifying the fat finally by filtration. To ensure that the sample obtained was truly representative of the whole, tests were always made to ensure that the aqueous phase never contained more than the merest trace of fat. The figures for the iodine and Reichert Meissl values are shown diagrammatically in Fig. 2, where very regular changes can be observed for the fat secreted by each of the three cows during the fast.

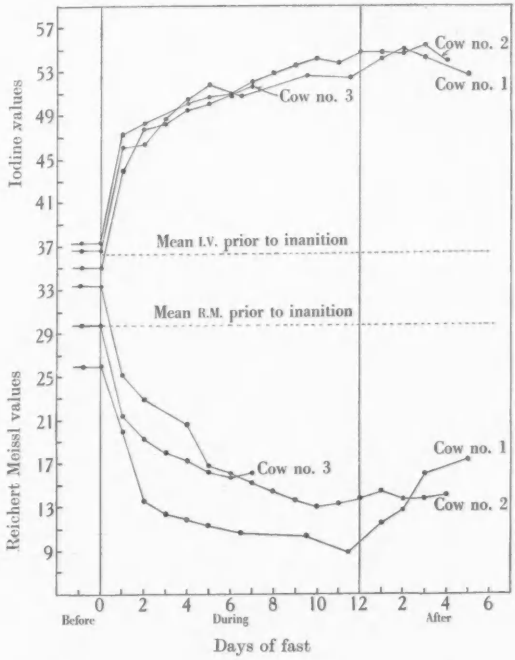

Fig. 2. The effect of inanition on the iodine values and Reichert Meissl values of the milk fat.

Analysis by fractional distillation. Owing to the complex nature of butter fat the analysis of its component fatty acids has always presented some difficulty. Two main procedures have been used. Hilditch & Jones [1929] and Hilditch & Thompson [1936] first saponify the fat and then distil the acidified products for some hours in a current of steam. This is followed by the fractional distillation of the steam-volatile compounds, while the non-volatile residue is separated by the Twitchell process into solid and liquid acids. These are then converted into methyl esters and fractionated. Owing to other sources of error which seem to be unavoidable in any process of this type which can at present be devised, this procedure probably gives results which are as accurate as possible, but the present authors believe that there are slight disadvantages in the actual technique. One of these lies in the fact that during steam distillation, the non-volatile fatty acids have to be heated to about 100° in the presence of steam for several hours, a process which ought to be avoided when unsaturated acids are

present; another is that, unless very large quantities of the original butter fat are available, the amount of volatile compounds resulting from the steam distillation must be exceedingly small for their accurate fractionation. In the modified method now suggested distillation in steam is dispensed with, and the lower acids can be estimated more readily on smaller amounts of initial material. Bosworth & Brown [1933] also avoided the use of steam distillation in making a detailed investigation of the lower components of butter fat and were thus able to establish the presence of decenoic and tetradecenoic acids, but they did not adapt the method to the complete analysis of the total fatty acids. To do this the Twitchell separation of liquid and solid acids must be introduced at some stage in the process. The scheme which was therefore adopted in the present work may be summarized briefly by saying that the fat was converted directly into methyl esters, the lower components were fractionally distilled from the whole bulk, the higher members being separated into liquid and solid acids, methylated and also fractionally distilled. In order to explain the method more fully reference will be made in detail to the analysis of a typical butter fat, that secreted by Cow no. 1 prior to starvation.

The analysis of butter fatty acids by the modified method. The butter fat (about 287 g., Reichert Meissl value 26, I.V. 36·6) was first refluxed in 2 l. methyl alcohol containing 5 % H_2SO_4 for 24 hr. to convert the glycerides into methyl esters. Most of the alcohol was then boiled off and kept, while the esters were exhaustively extracted with ether and the united ether extracts washed three times with a little water. The ether solution was desiccated over sodium sulphate, filtered and the solvent almost completely removed in the water bath at ordinary pressure. The flask containing the esters was then connected to an electrically heated and packed fractionating column similar to that described by Longenecker [1937] and the last traces of solvent were carefully removed and collected under a very slight vacuum. Saponification values were then obtained for the methyl alcohol and ether was distilled from the esters at the two different stages, while the united aqueous portions were saponified with alkali, acidified with H_2SO_4, steam-distilled and the distillate was titrated. As all the methyl esters higher than the butyrate are insoluble in water it is reasonable to assume that all the acidity present in the various solvents was due to butyric acid. The amount of this compound present in the original fat could thus be calculated. A blank determination was carried out with a similar quantity of lard for which the Reichert Meissl value was negligible. The amounts of "butyric acid" estimated to be present for both the milk fat and the lard were 0·09 and 0·03 % in the aqueous portion, 2·73 and 0·03 % in the methyl alcohol and 0·68 and 0·25 % in the ether, making totals of 3·5 and 0·31 % of the butter and lard respectively. It is evident from these results that the estimated figure for butyric acid by this method tends to be somewhat high.

In the fractional distillation which followed, details of which appear in Table I, the first two fractions passed over at a pressure of 15 mm. and were kept separate. A large primary fraction containing all the esters distilling below 158–160° at 2 mm. was then collected, for it had been shown by preliminary trials that practically all the lower compounds up to and including most of the myristate pass over below that point, while the distillation of oleate is still relatively slight. This large primary fraction was then distilled into some 20 smaller fractions for which the iodine values and molecular weights were determined, and as these data suggested that the last fraction and residue consisted mainly of palmitate and oleate, these two final fractions were added again to the higher compounds still remaining undistilled. All these higher esters were

Table I. *The fractionation of the methyl esters prepared from 287 g. of the normal butter fat secreted by Cow no. 1 prior to inanition*

Fraction	B.P. at 2 mm. up to	% of total esters	Mol. wt.	I.V.	Fraction	B.P. at 2 mm. up to	% of total esters	Mol. wt.	I.V.
				Lower esters					
1	(Methyl butyrate)	3·88	—	—					
2	45*	0·35	132·1	0·6	12	118	0·76	216·4	9·1
3	45*	0·32	131·8	0·6	13	124	1·05	232·4	11·6
4	56	0·22	158·5	2·6	14	127	1·12	233·4	10·8
5	56	0·42	157·4	2·7	15	127	1·61	238·3	10·7
6	56	0·07	159·2	—	16	128	2·38	239·8	10·4
7	70–80	0·56	177·8	12·8	17	131	1·66	242·1	9·1
8	82	0·67	184·8	16·5	18	141	1·31	248·4	9·0
9	84	0·61	183·1	16·4	19	145	1·56	260·9	8·9
10	102	0·51	195·5	12·9	20	148	3·44	266·2	9·5
11	106	0·94	209·5	7·9	21	149	1·76	269·5	11·3
						Total	25·20		
				Solid esters					
22	140	0·45	245·0	0·7	29	162	3·16	281·0	8·2
23	149	0·67	262·3	0·4	30	158	3·25	283·2	9·0
24	153	1·61	268·4	1·3	31	161	2·73	284·0	9·8
25	153	2·38	274·4	4·0	32	165	2·92	289·2	11·2
26	159	2·93	274·5	4·1	33	165	1·93	290·2	13·7
27	157	3·47	277·8	5·0	34	170	2·69	297·3	13·9
28	161	3·20	278·0	5·6	35	Residue	3·05	307·7	17·9
						Total	34·44		
				Liquid esters					
36	132	0·30	237·2	17·1	44	163	3·27	287·8	77·6
37	132	0·46	244·2	17·4	45	164	2·76	288·7	79·9
38	145	0·37	254·9	27·4	46	165	2·78	290·5	82·1
39	153	0·60	268·9	46·3	47	166	4·72	295·1	85·9
40	155	1·20	281·5	67·9	48	168	4·91	295·3	87·4
41	155	2·40	282·2	72·2	49	168	4·26	295·5	88·7
42	161	2·18	284·7	72·4	50	170	4·47	294·8	90·3
43	163	2·40	287·1	76·6	51	Residue	3·28	303·1	93·1
						Total	40·36		

* At 15 mm. pressure.

then saponified and separated into liquid and solid acids and the liquid portion was freed from any small amounts of unsaponifiable matter which might be present. They were then esterified again and fractionally distilled through the electrically heated column. The iodine values, molecular weights and other data for the various fractions are recorded in Table I, the molecular weights being obtained either from the saponification values of the esters or by titration of the acids prepared from them. Corresponding data were then obtained for samples of starvation fat secreted by Cows 1 and 2, but in order to illustrate the marked differences between ordinary fat and that secreted during inanition, only the figures for the first of these need be recorded as shown in Table II.

Before the composition of the various fractions could be calculated, according to the method adopted and discussed by Irving & Smith [1935], it was first necessary to oxidize several fractions to ensure that the assumptions made as to their actual nature were justified. There is, however, no necessity to describe these oxidations in detail. In order to show the type of fractionation obtained without the use of steam distillation, the intermediate results obtained for the composition of the three main fractions, the lower, solid and liquid esters of both

Table II. *The fractionation of the methyl esters prepared from 285 g. of the butter fat secreted by Cow no. 1 on the 11th and 12th days of inanition*

Fraction	B.P. at 2 mm. up to	% of total esters	Mol. wt.	I.V.	Fraction	B.P. at 2 mm. up to	% of total esters	Mol. wt.	I.V.
				Lower esters					
1	(Methyl butyrate)	1·27	—	—					
2	30–40	0·05	164·7	—	6	140	0·47	242·8	24·9
3	70–90	0·13	178·0	14·4	7	145	0·45	258·8	22·2
4	90–120	0·15	193·9	15·0	8	150	0·74	267·0	21·3
5	120–130	0·20	222·3	20·8	9	153	1·25	270·6	21·1
					10	157	1·11	275·4	29·0
						Total	5·82		
				Solid esters					
11	147	0·26	250·9	2·5	18	154	2·86	279·8	4·4
12	150	0·74	262·4	0·8	19	160	1·81	282·0	6·3
13	152	1·79	269·0	1·0	20	165	3·32	294·2	9·9
14	152	3·50	271·7	2·0	21	167	4·06	298·0	11·1
15	153	3·15	275·6	3·0	22	168	3·55	300·4	11·8
16	153	4·25	275·2	2·1	23	Residue	3·08	303·7	14·9
17	154	3·86	277·3	3·5		Total	36·23		
				Liquid esters					
24	140	0·33	243·6	40·9	33	170	5·17	294·0	86·1
25	150	0·46	260·0	49·2	34	175	1·30	293·0	87·5
26	160	0·96	274·6	61·6	35	180	2·47	294·3	86·9
27	164	1·49	288·3	79·3	36	180	7·16	296·2	87·4
28	166	1·59	291·6	83·0	37	180	8·12	296·8	89·2
29	170	2·08	291·1	83·2	38	182	7·63	297·8	90·1
30	170	1·46	294·2	84·0	39	182	4·95	299·0	91·5
31	170	2·47	294·7	85·3	40	Residue	6·20	306·0	91·9
32	170	4·11	294·4	85·9		Total	57·95		

Table III. *Cow no. 1, the composition of the lower, liquid and solid fractions before and during inanition*

	Methyl esters of the normal fat secreted before inanition				Methyl esters of the fat secreted on the 11th and 12th days of inanition			
Fraction	Lower	Liquid	Solid	Total	Lower	Liquid	Solid	Total
Saturated:								
C_4	3·88	—	—	3·88	1·27	—	—	1·27
C_6	0·64	—	—	0·64	—	—	—	—
C_8	0·98	—	—	0·98	0·11	—	—	0·11
C_{10}	1·85	—	—	1·85	0·18	—	—	0·18
C_{12}	2·51	0·05	—	2·56	0·14	—	—	0·14
C_{14}	8·07	3·08	0·74	11·89	1·00	1·25	0·53	2·78
C_{16}	5·32	0·31	17·83	23·46	1·96	0·23	17·84	20·03
C_{18}	—	0·15	11·38	11·53	—	0·04	14·27	14·31
C_{20}	—	—	1·08	1·08	—	—	0·89	0·89
Unsaturated:								
C_{10}	0·24	—	—	0·24	—	—	—	—
C_{12}	0·24	—	—	0·24	—	—	—	—
C_{14}	0·76	0·18	—	0·94	0·19	0·25	—	0·44
C_{16}	0·71	2·44	—	3·15	0·28	1·17	—	1·45
Oleic	—	32·16	3·41	35·57	0·69	49·23	2·70	52·62
Linoleic	—	1·17	—	1·17	—	2·47	—	2·47
C_{20}	—	0·82	—	0·82	—	3·31	—	3·31
Total	25·20	40·36	34·44	100·00	5·82	57·95	36·23	100·00

Table IV. *The component fatty acids of the different butter fats expressed in weight and molar percentages*

	Wt. per 100 g.			Molecules per 100 molecules		
	Cow no. 1		Cow no. 2	Cow no. 1		Cow no. 2
Acid	Before inanition	During inanition*	During inanition†	Before inanition	During inanition*	During inanition†
Saturated:						
C_4	3·5	1·2	2·7	9·7	3·5	7·9
C_6	0·6	—	0·1	1·2	—	0·1
C_8	1·0	0·1	0·1	1·6	0·2	0·2
C_{10}	1·8	0·2	1·0	2·5	0·3	1·5
C_{12}	2·5	0·1	0·6	3·0	0·2	0·7
C_{14}	11·9	2·8	3·8	12·5	3·2	4·3
C_{16}	23·5	20·0	22·1	22·1	20·9	22·1
C_{18}	11·6	14·3	9·9	9·8	13·5	8·9
C_{20}	1·1	0·9	0·9	0·8	0·8	0·8
Total	57·5	39·6	41·2	63·2	42·6	46·5
Unsaturated:						
C_{10}	0·2	—	0·2‡	0·3	—	0·2
C_{12}	0·2	—	0·2‡	0·3	—	0·3
C_{14}	0·9	0·4‡	0·4‡	1·0	0·5	0·5
C_{16}	3·2	1·4‡	2·0	3·0	1·5	2·0
Oleic	35·9	52·8	51·7	30·5	50·1	46·9
Linoleic	1·2	2·5	0·8	1·0	2·4	0·7
C_{20}	0·8	3·3	3·5	0·6	2·9	2·9
Total	42·4	60·4	58·8	36·7	57·4	53·5
Total of all the acids up to C_{14}	22·6	4·8	9·1	32·1	7·9	15·7

* A sample obtained by mixing the fat secreted on the 11th and 12th days of inanition.
† A pooled sample from the fat secreted on the last 6 days of inanition.
‡ Where the amount of lower acids present is very low and oleic acid predominates as in the fats of inanition, it is probable that the figures marked ‡ are somewhat higher than the true values.

normal and "fasting" fat are arranged in Table III, while the final figures for all three fats are recorded in Table IV.

Linoleic acid. As it was at first thought that inanition might cause marked changes in the amount of linoleic acid present in the different fats, its presence was sought by oxidation methods in various fractions, but in no case could tetrahydroxystearic acid be isolated. This was doubtless due to the fact suggested by the final results of the fractionation that the linoleic acid content of the milk fat from these particular cows was as low as 1–2 % and that the amount of fat available was limited. But as the presence of octadecadienoic acid in normal butter fat has been shown beyond dispute by Green & Hilditch [1935], there can be little doubt that it also existed in smaller amounts in the present case.

Arachidonic acid. The presence of small amounts of arachidonic or some such highly unsaturated acid in the milk fat both before and during inanition was shown by brominating both the last fraction and the residue in the distillation of the liquid esters. The amount of bromo-derivative (Br 65·5 %, M.P. 228° with decomposition), obtained in both cases was less than 0·1 % of the whole.

Discussion

The yields of fat. For all three animals the period of inanition was characterized by a very considerable decrease in the daily secretion of milk fat. Thus it can readily be seen from Fig. 1 that for the first cow, in which inanition was not

complicated by other factors, the yield fell quickly from 568 g. before the fast to 353 g. on the 3rd day, and then more slowly to a mean of 227 g., or 40 % of the original during the last 3 days of the fast. On re-alimentation a further slight drop was noticeable, but 5 days later the daily yield began to increase and at the end of about 6 weeks it had returned to some 60 % of the pre-fast value. With Cow no. 2, in which milk fever symptoms developed on the 3rd day, the secretion of fat and milk almost ceased while this complication lasted, but by the 5th day, the fat production had risen to 12 %, and before the end of the fast to 20 % of the original value. On re-alimentation the yield followed a course almost parallel with that of Cow no. 1 during the same period.

The composition of the fats. Reference to Fig. 2 shows clearly that inanition caused a marked progressive change in the nature of the fats secreted by each of the three cows as judged by their iodine and Reichert Meissl values, and that these changes were similar for all three animals. It will be observed that the initial iodine values of 36·6 and 37·1 for Cows 1 and 2 rose quickly to 46·1 and 47·3 on the 1st day of the fast, and then more slowly to 52·5 and 54·9 by the 12th day, the figures for Cow no. 3 following a similar course till the 7th day when this particular animal was withdrawn from the experiment. Then again the Reichert Meissl values for Cows 1 and 2 fell rapidly in the first 2 days from 26·0 and 33·3 to 14·5 and 22·9 and then still further to 9·8 and 13·8 by the end of the fast. On re-alimentation a slight tendency to return to normal was observed in both values after the first few days and this tendency continued somewhat spasmodically for the next few weeks until figures similar to the pre-fast values were attained. These results suggest that inanition causes a considerable increase in the content of unsaturated acids and a marked fall in that of lower fatty acids normally present as glycerides in butter fat. This was confirmed by more detailed analyses based on fractional distillation methods, for reference to the totals recorded in Tables I and II shows that while the proportion of higher solid esters remained almost unaffected as a result of inanition, the lower esters were reduced from a pre-fast value of 25·2 to 5·8 % during inanition, and that the corresponding figures for the liquid esters showed an almost equal increase from 40·4 to 58 %. Again it will be observed from the weight percentages in Table IV that the sum of all the acids up to and including C_{14} amounted to 22·6 % before the fast and to no more than 4·8 % during inanition, a decrease of 17·8 units, while the oleic acid content rose by 16·9 units from 35·9 to 52·8 %. Calculated also on a molar basis, as shown in Table IV, the fall of 24·2 molecules % in the lower acids is mainly accounted for by a rise of almost 20 molecules % in the oleic acid. It is therefore evident that the chief effect of inanition is the replacement of about 80 % of the lower constituents of the fat mainly by oleic acid, and that the whole of this decrease is distributed over all the lower members from C_4 to C_{14}, although the first and last of these appear to suffer least. Thus the butyric acid content only fell from 3·5 to 1·2 % a trifle more than a third of the original, the myristic by a somewhat similar amount and the others to less than an eighth of their pre-fast values. Of the other saturated acids, the amount of palmitic fell slightly from 23·5 to 20·0 %, while the stearic increased from 11·6 to 14·3 %. That the latter rise had probably some significance is shown best by reference to the molar percentages, where it will be observed that stearic acid increased from 9·8 to 13·5 %, almost a 40 % increase, whereas the fall in palmitic acid only amounted to some 5 % of the pre-fast value. In fact the constancy of the palmitic acid in all three sets of results is worthy of particular notice. The saturated acids represented as C_{20} also remained virtually unchanged. In the unsaturated acids higher than C_{14} and other than oleic, the chief change appeared to be a decrease

in palmitoleic acid from 3·2 to 1·4% and a most marked rise from 0·8 to 3·3% in higher unsaturated components represented as "C_{20}".

The results for the fat secreted by Cow no. 2 during inanition are also recorded in Table IV, where it will be seen that with the probable exception of stearic and linoleic acids, the general effect of the fast was the same for both animals.

The relationship between the lipoids of the blood and milk. In discussing the relationship between the blood lipoids and the milk fat it must be remembered that at present there is no means of analysing the triglyceride fatty acids of the blood by themselves but only when mixed with those originally present as cholesteryl esters. There is therefore every possibility that the triglyceride acids of the blood may change and yet the change may be impossible to detect, and as these acids probably form one of the most important sources of milk fat, the disadvantage inherent in this fact for research work of the present type cannot be overestimated. At the same time, bearing this limitation in mind, it is well worth observing the almost complete absence of change already noted in the blood fatty acids and particularly in those of the non-phosphatide fraction of the plasma during inanition as compared with the very striking alterations brought about in the fat of the milk. In fact, the latter suggest that the normal equilibrium existing between the amount of fat actually formed in the gland and that arising directly from the blood was very considerably disturbed. For example, the enhanced concentration of higher unsaturated compounds of the C_{20} type during the fast might well be taken as indicating that there was an increase in the unsaturated acids arising directly from the blood in which the proportion of these acids has been shown by Parry & Smith [1936] to be exceedingly high, and that the proportion of fat synthesis in the gland was considerably decreased.

The greatest change, however, was in the amount of lower fatty acids present and these are worthy of special consideration. With regard to their source in normal butter fat there are probably three main possibilities. In the first place, they may pre-exist in the blood and arise from it directly. Secondly, they may be formed in the mammary gland itself by breakdown of oleo-glycerides as suggested by Hilditch & Thompson [1936] and by Hilditch & Paul [1936]. Thirdly, they may arise, again in the gland itself, as by-products in the synthesis of the fat from some form of carbohydrate material. The theory that they may arise directly from the blood gains support from the suggestion that lower fatty acids may be formed as by-products of rumination in the cow and that these may then be transported by the blood to the mammary gland. In human milk the content of these lower constituents is exceedingly small. From the present work this theory might also appear to receive confirmation from the fact that when rumination ceased as a result of the enforced fast, the lower fatty acid content of the milk also fell. In this connexion two points should, however, be noted. In the first place it would be natural for these lower compounds, if they exist in blood, to be present in the non-phosphatide fraction of the plasma. But the results recorded in Table II of the previous paper (p. 1861) suggest that if any of these lower acids were present as esters in this fraction, their amount must have been very small, and that they certainly did not appear to decrease during inanition. It is always possible, however, that during the analysis of the blood lipoids free water-soluble acids were lost, for such an occurrence would be exceedingly difficult either to detect or avoid. In the second place, if the reduction in these lower constituents of milk fat were due in the present instance to a deficiency of the same acids in the blood, it would be natural to expect that their decrease in the milk fat during inanition would be counterbalanced by increases fairly evenly distributed over the other constituents, whereas it has already been

shown that almost the whole loss is made good by oleic acid alone. Indeed this last observation might well be taken as evidence in support of Hilditch's suggestion that the lower acids arise by the oxidation of oleo-glycerides in the gland itself, for if this were so, the catabolism of these glycerides might not be so essential during inanition when the general activity of the gland was considerably reduced, with the consequence that the content of the lower constituents would fall and that of oleic acid rise. Recent work, however, by Graham *et al.* [1938], based partly on respiratory quotient measurements, has definitely suggested that fat metabolism in the mammary gland probably takes the form of synthesis rather than of breakdown. The explanation of the present results may therefore be that the lower compounds are formed as by-products in the synthesis of oleic acid, but that during inanition, when the gland is producing much less than its usual amount of fat, the smaller amount of synthesis taking place is able to proceed to its final stages, so that the lower acids are almost completely converted into oleic. This suggestion gains support from the well-known fact that diets rich in carbohydrates and poor in fat cause an increase in the lower fatty acids of the milk fat, while those poor in carbohydrates and rich in fat have the reverse effect, indicating that with diets on which fat synthesis would probably be most necessary the lower fatty acids increase. At present it is impossible to decide as to which of these various theories is correct but it is hoped that future work now contemplated will throw more light on this particular aspect of the subject. At present the fact of chief importance emerging from the results just described is the inverse relationship existing between the oleic acid on the one hand and the lower acids on the other.

Summary

1. Although in the previous paper no appreciable change was observed during inanition in the nature of the blood lipoids of three lactating cows, very considerable changes have been found in the milk fat, and these have been studied in detail.

2. The chief change was a decrease of about 80 % in the original content of lower acids up to and including C_{14}, a deficiency which was almost entirely made good by an increase in the content of oleic acid.

3. The fact that oleic acid tended so completely to replace the lower constituents has been discussed in connexion with different theories previously put forward to explain the occurrence of these lower homologues in milk.

4. A description is given of a modified procedure for the analysis of butter fat by the fractional distillation method from which prolonged distillation of the saponified fat in steam is omitted.

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CCXLII. ENZYMIC PROTEOLYSIS

I. LIBERATION OF AMMONIA FROM PROTEINS[1]

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(*Received 21 July 1938*)

The isolation of asparagine [Damodaran, 1932] and of glutamine [Damodaran *et al.* 1932] from enzymic digests of proteins has given direct demonstration of the existence of amide groups in the protein molecule. Before, however, any attempt can be made to decide if the ammonia, which is an invariable product of protein hydrolysis, arises solely from dicarboxylic acid amides, it becomes essential to have more exact information than is at present available on the deamidizing activity of the proteolytic enzymes. Such information is in the first place necessary to enable a proper choice of enzymes and conditions of experiment to be made, so that digestion of protein could be effected with the least amount of decomposition of the ammonia-yielding complexes. Secondly, comparative study of the liberation of ammonia from proteins and from synthetic substrates is likely to provide analogies from which the nature of the precursors of ammonia might be inferred.

The rate of liberation of ammonia in tryptic digests of casein, gliadin and Witte's peptone was studied by Hunter & Smith [1925], who found that the

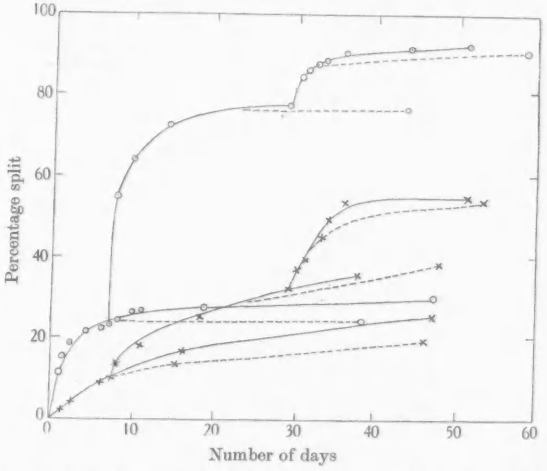

Fig. 1. Digestion of casein. ⊙ Amino. × Amide. — Digest. ---- Control.

formation of ammonia proceeded much more slowly than the liberation of amino groups. According to these authors "the differences were so great as to suggest

[1] Read before the Indian Science Congress, January 1938.

that the two processes are really catalysed by different enzymes and that pure trypsin might produce from proteins no ammonia at all".

As a preliminary to the work already cited on the isolation of asparagine and glutamine, a number of experiments were carried out on the splitting of

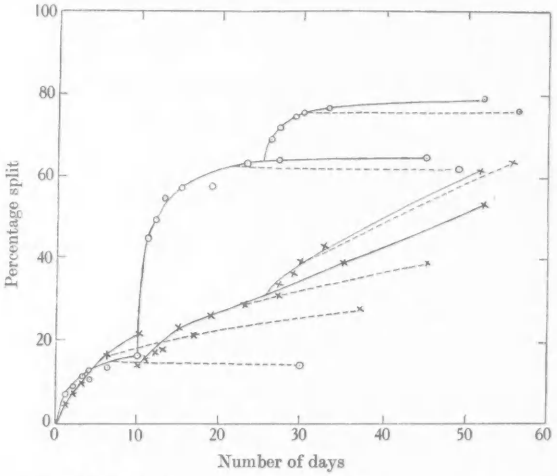

Fig. 2. Digestion of gliadin. ⊙ Amino. × Amide. — Digest. ---- Control.

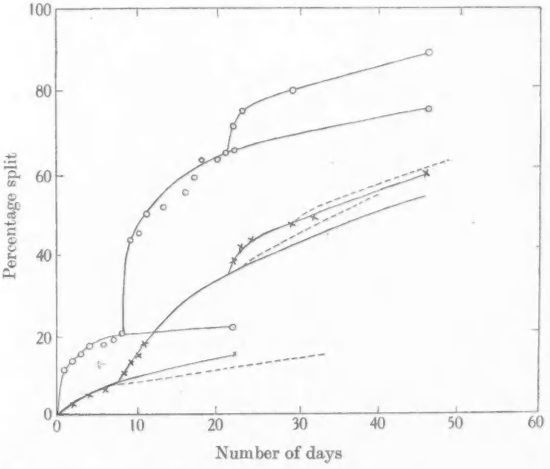

Fig. 3. Digestion of edestin. ⊙ Amino. × Amide. — Digest. ---- Control.

amide and peptide bonds during the action of proteolytic enzymes on gliadin and edestin. Attention was, however, mainly directed to securing the conditions under which a far-reaching hydrolysis of protein could be effected with minimal

liberation of ammonia and no conclusions were drawn as to any relation between proteolytic and deamidizing actions of the enzymes used.

The present paper describes a systematic study of the formation of ammonia and the hydrolysis of peptide linkages during the action of pepsin, trypsin, erepsin and papain on three typical proteins, casein, edestin and gliadin. Figs. 1–3 represent graphically the whole course of the digestion of each of these proteins by the three animal proteases acting in succession. The numerical data and details of procedure are given in Tables I, II, III. It will be seen from the curves, which show the progressive liberation of amino groups and of free ammonia during the course of digestion, that the rates of splitting in the two cases do not by any means run parallel. With each enzyme, in the earlier stages of action

Table I. *Pepsin, trypsin and erepsin on casein*

A. 22 g. casein (N 15·68 %) dissolved in about 500 ml. *N*/10 HCl + 475 ml. water + 1 g. B.D.H. pepsin dissolved in 25 ml. water.

B. 60 ml. of A (at the end of 8 days) boiled at *p*H 6 to inactivate pepsin, brought back to *p*H 1·6 and made up to 100 ml.

C. 450 ml. of A (at the end of 7 days) boiled at *p*H 6, brought to *p*H 8·6 with NaOH, 25 ml. of a solution of 0·5 g. of B.D.H. trypsin and sufficient water to make up to 500 ml.

D. 30 ml. of C (at the end of 15 days on trypsin) boiled at *p*H 6, adjusted to *p*H 8·6 and made up to 50 ml.

E. 140 ml. of C (after 22 days on trypsin) inactivated at *p*H 6, brought to *p*H 7·6 and made up to 250 ml. after the addition of 15 ml. of erepsin solution. After inactivation and before addition of erepsin the digest had to be left in the ice-chest for about 3 weeks.

F. 30 ml. of E boiled at *p*H 6 and made up to 50 ml. after adjusting to *p*H 7·6.

	Hydrolysis %											
	Pepsin				Trypsin				Erepsin			
	A		B (after inactivation)		C		D (after inactivation)		E		F (after inactivation)	
Time hr.	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide
0	—	—	—	—	27·30	8·50	—	—	79·70	35·00	—	—
1	33·29	—	—	—	—	—	—	—	—	—	—	—
2	3·43	—	—	—	39·10	8·50	—	—	80·80	35·00	—	—
4	6·57	—	—	—	46·10	—	—	—	—	—	—	—
6	9·43	—	—	—	—	—	—	—	81·30	35·00	—	—
8	10·42	—	—	—	54·05	—	—	—	—	—	—	—
12	11·00	0·80	—	—	55·60	—	—	—	—	—	—	—
days												
1	15·29	1·60	—	—	62·10	10·10	—	—	84·60	37·20	—	—
2	18·43	3·06	—	—	63·60	11·70	—	—	85·80	39·70	—	—
3	—	—	—	—	—	—	—	—	86·40	43·60	—	—
4	21·00	4·95	—	—	67·10	14·30	—	—	87·30	44·20	—	—
5	—	—	—	—	70·30	16·90	—	—	88·90	49·30	—	—
6	21·86	6·82	—	—	—	—	—	—	—	—	—	—
7	23·30	8·29	—	—	—	—	—	—	90·70	53·60	90·80	53·10
8	24·10	9·49	24·25	8·62	—	—	—	—	—	—	—	—
10	25·90	10·56	—	—	—	—	—	—	—	—	—	—
11	26·40	11·23	—	—	73·10	24·10	—	—	—	—	—	—
15	—	—	—	—	76·30	29·30	76·70	29·70	—	—	—	—
16	27·57	16·31	24·25	12·82	—	—	—	—	—	—	—	—
22	—	—	—	—	77·90	33·10	—	—	92·90	57·40	90·80	57·60
31	—	—	—	—	—	—	76·70	37·60	—	—	—	—
47	30·13	25·00	24·25	18·84	—	—	—	—	—	—	—	—
49	—	—	—	—	—	—	76·70	45·30	—	—	—	—
66	—	—	—	—	—	—	76·70	52·20	—	—	—	—

Table II. *Pepsin, trypsin and erepsin on gliadin*

A. 11 g. gliadin (N 17·25 %) suspended in about 400 ml. of *N*/20 HCl and treated with 0·5 g. of pepsin dissolved in a few ml. of *N*/20 HCl; clear solution obtained after incubation for 3 hr.

B. 30 ml. of A boiled at *p*H 6 on the 7th day, *p*H brought again to 1·6 and made up to 50 ml.

C. 350 ml. of A on the 10th day boiled at *p*H 6, brought to *p*H 8·6, 0·4 g. B.D.H. trypsin added and made up to 500 ml.

D. 30 ml. of C (after 20 days on trypsin) boiled at *p*H 6, reaction adjusted to *p*H 8·6 and made up to 50 ml.

E. 175 ml. of C (after 16 days on trypsin) boiled at *p*H 6, adjusted to *p*H 7·8 and 25 ml. of erepsin solution added.

F. 30 ml. of E boiled at *p*H 6 after 3 days on erepsin, *p*H readjusted to 7·8 and made up to 50 ml.

	Hydrolysis %											
	Pepsin				Trypsin				Erepsin			
	A		B (after inactivation)		C		D (after inactivation)		E		F (after inactivation)	
Time	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide
hr.												
0	—	—	—	—	16·40	15·12	—	—	63·50	29·00	—	—
1	—	—	—	—	23·70	15·12	—	—	—	—	—	—
2	—	—	—	—	30·16	15·12	—	—	65·50	29·00	—	—
4	—	—	—	—	35·60	15·12	—	—	66·10	29·00	—	—
6	—	—	—	—	38·30	15·12	—	—	67·30	29·00	—	—
7	0·81	0·04	—	—	—	—	—	—	—	—	—	—
10	2·24	0·90	—	—	—	—	—	—	—	—	—	—
12	—	—	—	—	43·10	15·29	—	—	67·50	29·90	—	—
days												
1	6·52	4·05	—	—	44·50	15·70	—	—	69·20	30·50	—	—
2	8·56	7·70	—	—	49·00	16·29	—	—	72·00	33·20	—	—
3	10·52	10·68	—	—	54·30	17·64	—	—	—	—	73·30	39·20
4	12·16	12·69	—	—	—	—	—	—	74·40	36·50	—	—
5	—	—	—	—	56·70	23·18	—	—	75·50	39·20	—	—
6	13·72	16·38	—	—	—	—	—	—	—	—	—	—
7	—	—	14·10	16·55	—	—	—	—	—	—	—	—
8	—	—	—	—	—	—	—	—	76·10	43·10	—	—
9	—	—	—	—	57·70	25·20	—	—	—	—	—	—
10	16·54	21·60	—	—	—	—	—	—	—	—	—	—
11	—	—	—	—	—	—	—	—	—	—	73·30	48·00
13	—	—	—	—	63·60	28·40	—	—	76·50	45·60	—	—
17	—	—	14·10	20·30	64·00	30·41	—	—	—	—	—	—
20	—	—	—	—	—	—	64·60	28·34	—	—	—	—
27	—	—	—	—	—	—	—	—	78·80	60·80	—	—
32	—	—	—	—	—	—	64·60	38·59	—	—	73·30	60·70
49	—	—	—	—	—	—	64·60	54·04	—	—	—	—

when the rate of peptide hydrolysis is at its highest, ammonia liberation is hardly noticeable and becomes significant only after the products of digestion have accumulated to some extent. On the other hand when the most active stage of proteolysis is past and formaldehyde titrations are increasing only very slowly, as indicated by the flattening of the amino-N curves, the liberation of ammonia continues at a steady rate and shows no tendency to slow down. These results are clearly brought out in Table IV and Figs. 4 and 5, in which the extents of amide and peptide hydrolysis at intervals at the beginning and end of the digestion are compared. The observations are readily explicable on the assumption that the ammonia formed is, at least in great part, not directly connected with

Table III. *Pepsin, trypsin and erepsin on edestin*

A. 17·5 g. of crystalline edestin stirred up with 375 ml. of *N*/10 HCl and kept at 37° for 1 hr., 350 ml. of water and 0·75 g. of pepsin in 25 ml. added to the clear solution.

B. 30 ml. of A boiled at *p*H 6 on the 18th day brought to *p*H 1·6 and made up to 50 ml.

C. 425 ml. of A on the 9th day boiled at *p*H 6 brought to *p*H 8·6, 0·5 g. B.D.H. trypsin added and made up to 500 ml.

D. 30 ml. of C boiled at *p*H 6 (after 15 days on trypsin) adjusted to *p*H 8·6 and made up to 50 ml.

E. 175 ml. of C (after 13 days on trypsin) boiled at *p*H 6 adjusted to *p*H 7·8 and 25 ml. of erepsin solution added.

F. 40 ml. of E boiled at *p*H 6 (after 17 days on erepsin), readjusted to *p*H 7·8 and made up to 50 ml.

	Hydrolysis %											
	Pepsin				Trypsin				Erepsin			
	A		B (after inactivation)		C		D (after inactivation)		E		F (after inactivation)	
Time hr.	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide
0	—	—	—	—	20·10	11·50	—	—	62·10	34·80	—	—
1	—	—	—	—	27·10	—	—	—	—	—	—	—
2	2·44	—	—	—	—	—	—	—	65·10	34·80	—	—
3	5·49	—	—	—	31·80	11·50	—	—	—	—	—	—
4	—	—	—	—	—	—	—	—	65·50	—	—	—
5	—	—	—	—	33·40	—	—	—	—	—	—	—
6	7·93	—	—	—	—	—	—	—	66·40	—	—	—
8	—	—	—	—	37·80	11·80	—	—	—	—	—	—
12	10·54	0·34	—	—	—	—	—	—	—	—	—	—
days												
1	11·10	1·02	—	—	42·30	13·40	—	—	73·20	37·10	—	—
2	13·29	2·38	—	—	44·60	15·40	—	—	76·00	40·70	—	—
3	14·76	4·14	—	—	49·70	17·30	—	—	76·20	42·60	—	—
4	16·83	5·21	—	—	—	—	—	—	76·80	—	—	—
5	—	—	—	—	53·40	21·40	—	—	—	—	—	—
6	17·68	6·18	—	—	—	—	—	—	—	—	—	—
7	18·29	7·35	—	—	—	—	—	—	—	—	—	—
8	—	—	—	—	57·80	27·70	—	—	79·20	46·30	79·80	43·60
9	—	—	—	—	59·90	29·00	—	—	—	—	—	—
10	—	8·22	—	—	62·00	29·90	—	—	—	—	—	—
12	—	—	—	—	62·30	31·00	—	—	—	—	—	—
14	—	—	—	—	65·30	35·40	—	—	—	—	—	—
15	—	—	—	—	—	—	63·20	35·90	—	—	—	—
17	—	—	—	—	—	—	—	—	—	—	80·50	56·60
18	—	—	17·48	7·19	—	—	—	—	—	—	—	—
22	21·96	14·08	—	—	—	—	—	—	—	—	—	—
24	—	—	—	—	—	—	63·20	48·10	—	—	—	—
25	—	—	—	—	—	—	—	—	88·90	59·20	—	—
32	—	—	18·90	13·98	—	—	—	—	—	—	—	—
38	—	—	—	—	74·00	54·70	—	—	—	—	—	—
42	—	—	—	—	—	—	—	—	89·10	67·50	—	—
43	—	—	—	—	—	—	—	—	—	—	81·20	61·60
56	—	—	—	—	—	—	63·20	63·90	—	—	—	—
74	—	—	18·91	22·77	—	—	—	—	—	—	—	—

proteolytic action but arises from the decomposition of the primary products of protein cleavage similar to the γ-peptides of glutamine studied by Melville [1935]. It also seems safe to conclude that this secondary decomposition is not enzymic in nature but is brought about by the acidic or alkaline reactions involved. Thus when peptic, tryptic and ereptic digests, in which the increase in amino groups have progressed considerably, are boiled to inactivate the enzyme

and incubated the formation of ammonia goes on at approximately the same rate as in unboiled digests containing the active enzyme, although the former

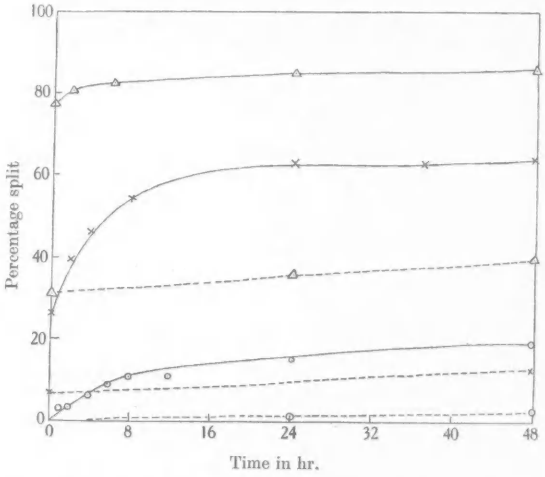

Fig. 4. Digestion of casein, initial stages. — Amino. --- Amide. ⊙ Pepsin. × Trypsin. ◬ Erepsin.

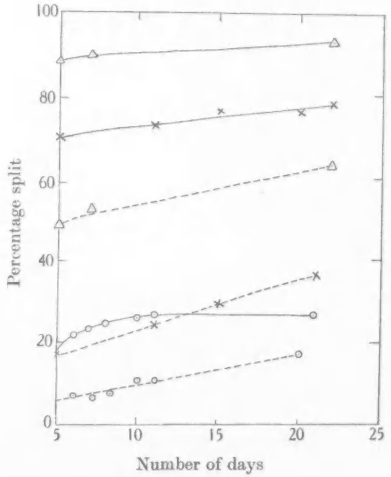

Fig. 5. Digestion of casein, final stages. ⊙ Pepsin. × Trypsin. ◬ Erepsin. ---- Amide. — Amino.

show no further increase in amino groups. Similar results were obtained with purified trypsin and erepsin (Fig. 6 and Table V).

Table IV

Protein	Enzyme	Interval in days: From	Interval in days: To	% hydrolysis in the interval: Peptide	% hydrolysis in the interval: Amide
Casein	(*a*) Pepsin	0	2	18·43	3·06
		8	47	6·03	15·51
	(*b*) Control	8	47	0·00	10·22
	(*a*) Trypsin	0	1	34·80	1·60
		15	22	1·60	3·80
	(*b*) Control	15	66	0·00	22·50
	(*a*) Erepsin	0	1	4·90	2·20
		7	22	2·20	3·80
	(*b*) Control	7	22	0·00	4·50
Gliadin	(*a*) Pepsin	0	2	8·56	7·70
		6	10	2·82	5·22
	(*b*) Control	6	36	0·00	10·44
	(*a*) Trypsin	0	2	32·60	1·17
		17	35	0·80	17·59
	(*b*) Control	17	46	0·00	25·74
	(*a*) Erepsin	0	2	8·50	4·20
		2	27	6·80	27·60
	(*b*) Control	3	31	0·00	21·50
Edestin	(*a*) Pepsin	0	2	13·29	2·38
		22	64	7·29	10·73
	(*b*) Control	18	74	1·33	15·58
	(*a*) Trypsin	0	2	24·50	1·90
		14	38	8·70	19·30
	(*b*) Control	15	56	0·00	28·00
	(*a*) Erepsin	0	2	13·90	5·90
		8	42	9·90	21·20
	(*b*) Control	8	43	1·40	17·50

Table VI gives the results of an interesting experiment in which the deamidizing action of pepsin was tested on casein previously digested with trypsin and erepsin. It is seen that pepsin is unable to bring about any further hydrolysis of peptide groups but that ammonia production takes place as in a peptic digest of native casein. Further, the amount of ammonia produced is not higher than in a portion of the trypsin-erepsin digest kept at the *p*H necessary for the action of pepsin, but without the addition of the latter. This experiment brings out another interesting result not, however, connected with deamidation. When casein is acted upon by pepsin, trypsin and erepsin in that order the extent of hydrolysis is nearly 90% of complete hydrolysis (about 20% due to pepsin and the remaining 70% due to the other two enzymes). When trypsin and erepsin are used first the splitting produced by them is still 70%, but pepsin is now unable to act further on this digest and contribute its quota to bring up the hydrolysis to the usual 90%. This result would suggest that the deciding factor for the action of pepsin is the position of the bonds attacked rather than their nature.

Table VII and Fig. 8 show the action of papain on casein, and Table VIII the action of papain on edestin. The results summarized in Table IX for the action of papain on casein are entirely different from those obtained with the animal proteases. It must be stated at the outset that, notwithstanding the

Table V. *Purified trypsin and erepsin on casein*

A. 15 g. casein dissolved in very dilute alkali, boiled for a minute and made up to 700 ml. at *p*H 8·6, 100 ml. of erepsin-free trypsin solution, also at *p*H 8·6, added.

B. 200 ml. of A on the 26th day boiled at *p*H 6 and after adjusting the reaction to *p*H 7·8, 25 ml. of trypsin-free erepsin added and the volume made up to 250 ml.

	Hydrolysis %			
	A Erepsin-free trypsin		B Trypsin-free erepsin	
Time hr.	Peptide	Amide	Peptide	Amide
0	—	—	48·70	27·31
1	3·50	—	—	—
2	6·10	—	50·44	—
6	—	—	55·60	27·52
days				
1	32·90	1·76	63·50	31·30
2	35·10	5·18	69·40	34·66
3	—	—	74·80	36·97
4	40·20	8·47	75·20	39·50
5	—	—	76·60	40·55
6	45·10	9·24	—	—
7	—	—	76·80	44·75
8	45·40	11·96	—	—
11	—	—	76·90	50·21
12	45·40	16·59	—	—
24	—	—	79·30	55·88
25	48·80	26·57	—	—

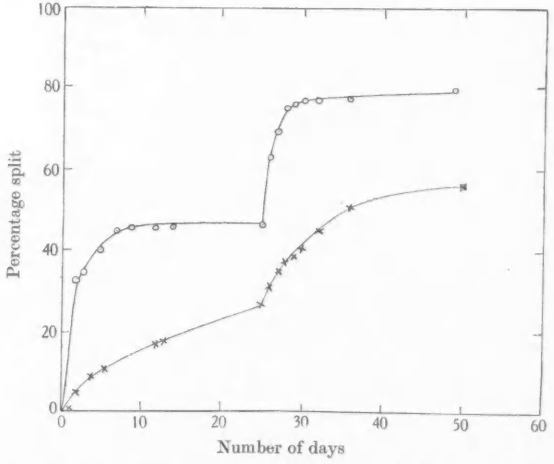

Fig. 6. Purified trypsin and erepsin on casein. ⊙ Amino. × Amide.

statements in the literature that activated papain is able to act on natural proteins, we have never succeeded in getting any of the proteins used in these experiments into solution by the action of papain-HCN either with commercial samples or with the enzyme prepared from the fresh latex. In all experiments

Table VI. *Pepsin after trypsin and erepsin on casein*

A. 22 g. casein dissolved in dilute NaOH, *p*H adjusted to 8·6, 1 g. B.D.H. trypsin added and made up to 1 l.

B. 800 ml. of A on the 8th day boiled at *p*H 6, made up to 1 l. at *p*H 7·8, with 100 ml. of erepsin solution and water.

C. 400 ml. of B on the 9th day inactivated by boiling at *p*H 6 brought to *p*H 1·8 with *N* HCl and made up to 500 ml. 250 ml. pipetted out and 0·25 g. of B.D.H. pepsin powder stirred in.

D. 250 ml. of C without the addition of pepsin.

	Hydrolysis %							
	Trypsin A		Erepsin B		Pepsin C		Pepsin D	
Time hr.	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide
0	—	—	46·38	14·25	72·90	30·08	—	—
1	14·10	—	—	—	72·90	30·31	—	—
2	—	—	49·19	—	72·90	30·31	—	—
4	—	—	50·66	14·25	72·90	31·09	—	—
6	—	—	—	—	72·90	31·20	—	—
7	25·10	0·27	—	—	—	—	—	—
days								
1	35·60	0·95	59·57	16·65	72·90	35·75	72·90	35·88
2	39·20	4·89	62·91	19·27	72·90	41·40	72·90	40·56
3	—	—	—	—	72·90	44·35	72·90	42·04
4	43·70	8·98	70·52	23·27	—	—	—	—
5	45·70	9·93	71·25	—	—	—	—	—
6	—	—	72·56	29·25	—	—	—	—
7	48·20	13·56	—	—	72·90	48·37	72·90	47·46

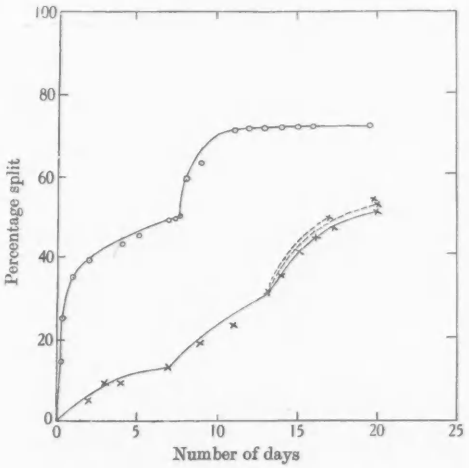

Fig. 7. Pepsin after trypsin and erepsin. ⊙ Amino. × Amide. — Digest. - - - - Control.

Table VII. *Papain and papain-HCN on casein*

Casein digested with pepsin-HCl for 24 hr. and inactivated by boiling at *p*H 5.
A. 200 ml. of casein peptone and 0·225 g. papain powder in 50 ml. of water.
B. 40 ml. of A boiled on the 34th day and made up to 50 ml.
C. 200 ml. of casein peptone and 50 ml. of papain-HCN (obtained by adding 10 ml. of 3% KCN solution neutralized to methyl red with HCl and incubating at 37° for 1 hr).
D. 80 ml. of C boiled after 15 days and made up to 100 ml.

	Hydrolysis %							
	Papain				Papain-HCN			
	A		B		C		D	
Time	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide
hr.								
0	13·55	8·81	—	—	13·55	8·81	—	—
5	—	—	—	—	31·25	18·84	—	—
days								
1	21·43	18·62	—	—	43·78	29·64	—	—
2	23·82	19·15	—	—	47·06	32·66	—	—
4	24·33	21·24	—	—	53·21	36·85	—	—
6	25·00	22·56	—	—	56·36	38·56	—	—
9	—	—	—	—	60·39	41·71	—	—
10	—	—	—	—	—	—	63·81	41·30
15	—	—	—	—	62·63	44·27	—	—
16	31·23	26·89	—	—	—	—	—	—
17	—	—	—	—	—	—	63·81	41·62
34	33·36	28·60	33·40	28·62	68·92	48·93	—	—
35	—	—	—	—	—	—	63·81	44·25
41	—	—	33·40	28·77	—	—	—	—
59	—	—	33·40	30·16	—	—	—	—

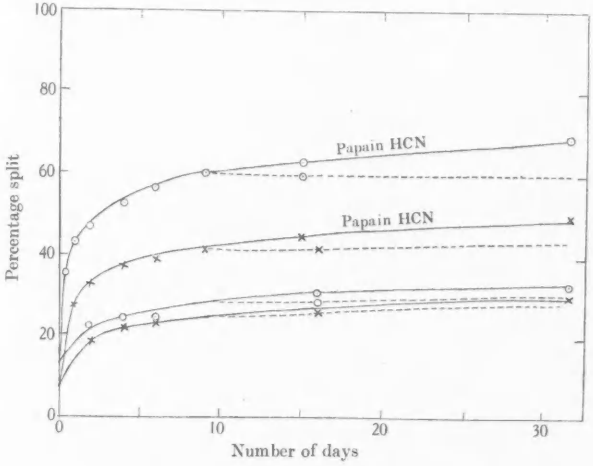

Fig. 8. Casein peptone + papain. ⊙ Amino. × Amide.
— Digest. ---- Boiled control.

Table VIII. *Papain and papain-HCN on edestin*

Edestin digested with pepsin-HCl for 24 hr.
A, C and D prepared from edestin peptone as in Table VIII.

	Hydrolysis %					
	Papain A		Papain-HCN C		Papain-HCN D	
Time	Peptide	Amide	Peptide	Amide	Peptide	Amide
hr.						
0	12·96	0·64	12·96	0·64	—	—
2	—	—	18·68	4·67	—	—
5	—	—	23·40	7·51	—	—
days						
1	19·36	6·05	34·51	13·90	—	—
2	21·80	6·87	41·08	18·05	—	—
5	—	—	47·57	22·22	—	—
9	22·22	8·27	52·36	26·83	52·40	26·90
13	—	—	55·55	28·03	—	—
27	—	—	—	—	52·40	29·00

Table IX

	Interval in days		% hydrolysis in the interval	
Enzyme, etc.	From	To	Peptide	Amide
Casein peptone and papain-HCN	0	1	30·23	20·83
	1	15	18·85	14·63
	15	34	6·29	4·66
Control	9	34	0·00	2·95
Casein peptone and papain	0	1	7·88	9·81
	1	16	9·78	8·27
	16	34	2·15	1·71
Control	9	34	0·00	1·54

with papain, therefore, substrates used were proteins which were brought into solution by a 24 hr. digestion with pepsin. There is no doubt that in papain digests ammonia formation is connected with enzyme action. There is a certain parallelism between amide and peptide hydrolyses and further, activation by HCN accelerates the liberation of both amino groups and of free ammonia. The ammonia formed by secondary effects of the reaction medium in boiled digests is only a small fraction of the total ammonia produced during enzyme action. Further investigations are, however, necessary before it can be decided whether the power to hydrolyse peptide and amide bonds resides in the same enzyme or whether papain contains two components with those specific functions.

Experimental

Materials. The proteins used were prepared in the laboratory shortly before use. We have invariably found that commercial preparations of proteins are entirely unsuitable for quantitative digestion experiments on account of their physical properties which cause difficulty in getting them into solution or uniform suspension. The preparations were obtained by the usual methods, dehydrated with acetone without allowing prolonged contact, and the acetone removed rapidly by a current of air.

Enzymes. In most of the experiments B.D.H. preparations of pepsin and trypsin were used. In the series with purified enzymes erepsin-free trypsin (proteinase + carboxypolypeptidase) was prepared from defatted pig's pancreas by the method described by Waldschmidt-Leitz & Harteneck [1925]. Erepsin solution was obtained for ordinary experiments from an 87% glycerol extract of pig's intestinal mucosa by the following procedure, all operations involved being carried out at 0°. One volume of the glycerol extract was diluted with three volumes of ice-cold water and centrifuged. The supernatant was mixed with one-tenth its volume of $N/10$ acetic acid and again centrifuged to remove extraneous protein. The clear centrifugate was brought to pH 7·8 with alkali and immediately used. Trypsin-free erepsin (aminopolypeptidase + dipeptidase) was obtained from the crude solution obtained above by adsorption at pH 4·8 on aluminium hydroxide, C_γ, and elution with phosphate buffer at pH 8·3 [Waldschmidt-Leitz & Schaffner, 1926].

Papain used in these investigations was prepared in the laboratory from fresh papaya latex. The latex liberated by scarifying the skin of the fresh green fruits with a bone spatula was collected in a porcelain dish, the thick white fluid squeezed through muslin and precipitated by adding several volumes of ice-cold acetone. The precipitate was washed twice with acetone and dried over sulphuric acid *in vacuo*. A snow-white powder was thus obtained which dispersed in water to give a slightly opalescent solution. The preparation gave tests for peroxidase. For activation by HCN the method of Willstätter & Grassmann [1924] was used.

Preparation of digests. The digests were generally made up to contain 2 g. of protein and 0·1 g. of enzyme per 100 ml.

The starting of the digestion with pepsin was slightly different with the three proteins. With casein a solution in $N/10$ HCl could be readily obtained and after the addition of water and pepsin solution readings could be commenced immediately after mixing. Edestin suspended in $N/10$ HCl went into solution on being kept at 37° for 1 hr. with repeated shaking. Gliadin on the other hand formed a sticky mass on being suspended in $N/10$ HCl and went into solution only after digestion with pepsin for 3 hr. In all cases quantitative measurements were commenced only after homogeneous solutions were obtained. For digestion with papain the proteins were first brought into solution by means of pepsin which was then inactivated by boiling.

At the end of the action of pepsin, after leaving a small quantity for studying the effect of prolonged digestion, the main portion of the digest was brought to pH 6 and boiled to inactivate the enzyme. At this reaction it was found that boiling for 15 min. caused no appreciable increase in amino-N or ammonia. The solution was now cooled to 37° and, after the reaction was adjusted to pH 8·6 with NaOH, transferred to a measuring flask, mixed with a 5% trypsin solution containing one-twentieth the weight of the protein now present, and the volume made up at 37°. Readings were commenced immediately. A similar procedure was adopted in transferring the digest from trypsin to erepsin, 100 ml. of the latter being added to each 900 ml. of the inactivated tryptic digest. After the action of each enzyme had proceeded to a considerable extent a portion of the digest was inactivated and incubated to study the secondary formation of ammonia from the cleavage products under the influence of the reaction of the medium. The inactivation was brought about by boiling at pH 6, the reaction being then adjusted to the original pH of the particular digest.

Methods of analysis. On account of the difficulty of quantitatively dissolving a weighed amount of protein in a definite volume of liquid, calculation of percentage hydrolysis was made not on the basis of the weight of protein but on

the results of analysis of an aliquot of the digest subjected to complete hydrolysis by HCl and in which amino-N and ammonia-N were determined in the same way as in the enzyme digest. Such analyses were carried out after the addition of each enzyme and also in digests inactivated by boiling.

Nitrogen was determined by micro-Kjeldahl, amino nitrogen by Sørensen's formol titration (from *p*H 6·8 in aqueous solution to distinct pink with phenolphthalein in formalin). Free ammonia was determined by distillation *in vacuo* at 40° in the apparatus of Parnas & Heller [1924] but without the use of steam; a 10% solution of sodium carbonate was used for making the solution alkaline; it was found that distillation was always complete in 10 min. during which time 9–10 ml. of the distillate were collected.

Certain unavoidable errors in the methods of analysis have to be noted, viz. the interference in the formaldehyde titration by the ammonia present in the digest, and in the case of purified ereptic digests by phosphate in the buffer solution used for elution, as also the possible formation of ammonia from cyanide during vacuum distillation with sodium carbonate of papain-HCN digests. But experimentally and by calculation it could be shown that these errors are not of sufficient magnitude to affect the significance of the results obtained.

Summary

A study has been made of the progressive liberation of ammonia and of amino-groups during the action of pepsin, trypsin, erepsin and of papain on three typical proteins, casein, edestin and gliadin.

It is shown that amide and peptide hydrolyses do not by any means run parallel and that the ammonia formed is, at least in great part, not directly connected with proteolytic action but arises from the decomposition of the primary products of protein cleavage. This secondary decomposition is not enzymic in nature but is brought about by the acidic or alkaline reactions of the digestion media.

In papain digestion, on the other hand, ammonia formation appears to be an enzymic function. Papain either possesses deamidase activity itself or contains a component which has such activity.

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